

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 281 767 A2**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
05.02.2003 Bulletin 2003/06

(51) Int Cl.7: **C12Q 1/02, C12Q 1/66,**
G01N 33/50, A61K 49/00,
A61K 35/12, A61K 35/74,
A61K 35/76

(21) Application number: **01125911.6**

(22) Date of filing: **30.10.2001**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(72) Inventor: **Szalay, Aladar A.**
Highland, CA 92346-6213 (US)

(74) Representative: **Schüssler, Andrea, Dr.**
Kanzlei Huber & Schüssler
Truderinger Strasse 246
81825 München (DE)

(30) Priority: **31.07.2001 EP 01118417**

(71) Applicant: **Szalay, Aladar A.**
Highland, CA 92346-6213 (US)

(54) **Light emitting microorganisms and cells for diagnosis and therapy of tumors**

(57) Described are diagnostic and pharmaceutical compositions comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal, e. g. a luminescent or fluorescent protein. Moreover, the use of said microorganism or cell for tumor-targeting or

tumor-imaging is described. For therapeutic uses, said microorganism or cell additionally contain an expressible DNA sequence encoding a protein suitable for tumor therapy, e.g. a cytotoxic or cytostatic protein.

EP 1 281 767 A2

Description

[0001] The present invention relates to diagnostic and pharmaceutical compositions comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal, e.g. a luminescent or fluorescent protein. The present invention also relates to the use of said microorganism or cell for tumor-targeting or tumor-imaging. For therapeutic uses, said microorganism or cell additionally contain an expressible DNA sequence encoding a protein suitable for tumor therapy, e.g. a cytotoxic or cytostatic protein.

[0002] Presence of bacteria in tumors was reported approximately fifty years ago. Several publications substantiated the earlier clinical findings that unexpectedly large numbers of bacteria were discovered in excised tumors from human patients. Investigators argue that chronic infections may predispose cells to malignant growth. Chronic infections of various strains of *Chlamydia* have been associated with lung and cervical cancer as well as malignant lymphoma. Another well described association between the presence of a specific bacterial species and cancer development is *Helicobacter pylori* in patients with gastric ulcers. Elevated levels of *H. pylori*-associated antibodies have been found in patients with duodenal ulcer and gastric adenocarcinoma. These observations demonstrate a concomitant presence of bacteria at tumor sites; however, it was yet not clear whether the microorganisms were the cause of tumor formation or whether the tumorous tissues were more susceptible to bacterial colonization. Intravenously injected strict anaerobic bacteria, *Clostridium pasteurianum*, into mice replicated selectively in the tumor suggesting a hypoxic microenvironment in the necrotic center. Intravenous injection of attenuated *Salmonella typhimurium* mutants resulted in elevated bacterial titers in the tumor tissues in comparison to the other organs of mice upon histologic and bacteriologic analyses.

[0003] Similarly, the presence of virus particles was reported in excised human breast tumors as early as 1965. More recently, based on polymerase chain reaction (PCR) data, the human papillomavirus has been claimed to be associated with anogenital tumors and esophageal cancers, breast cancers, and most commonly, cervical cancers. In addition, the presence of hepatitis C virus in human hepatocellular carcinoma, Epstein-Barr virus in squamous cell carcinoma in Kirmura's disease, mouse mammary tumor virus-like particles (MMTV) in human breast cancer, SV40 virus in macaque astrocytoma, and herpesvirus in turtle fibropapilloma has also been reported. Surprisingly, the concentration of virus particles in the tumors shows variations among patients. The presence of human papillomavirus in squamous cell carcinomas of the esophagus ranges from 0 to 72% (10- 15). In contrast to tumor tissues, no virus particles have been found in tumor-free areas of the esophageal epithelium of the same patient suggesting that the virus particles are located only in the tumor tissues.

[0004] However, so far it could not undoubtedly been shown whether the above discussed microorganisms are responsible for the development of disorders like tumors (except for papillomaviruses) or whether, e.g., tumors can attract and/or protect viruses or bacteria. Accordingly, there was no basis for the use of such microorganisms for the diagnosis or therapy of tumors. Conventional tumor diagnostic methods, such as MRI (Magnetic Resonance Imaging) and therapeutic methods, e.g. surgery, are invasive and not very sensitive.

[0005] Therefore, it is the object of the present invention to provide a means for the efficient and reliable diagnosis as well as the therapy of tumors which overcomes the disadvantages of the diagnostic and therapeutic approaches presently used.

[0006] According to the present invention this is achieved by the subject matters defined in the claims. When *Vaccinia* virus (LIVP strain) carrying the light emitting fusion gene construct rVV-ruc-gfp was injected intravenously into nude mice, the virus particles were found to be cleared from all internal organs within 4 days, as determined by extinction of light emission. In contrast, when the fate of the injected *Vaccinia* virus was similarly followed in nude mice bearing tumors grown from subcutaneously implanted C6 rat glioma cells, virus particles were found to be retained over time in the tumor tissues, resulting in lasting light emission. The presence and amplification of the virus-encoded fusion proteins in the same tumor were monitored in live animals by observing GFP fluorescence under a stereomicroscope and by collecting luciferase-catalyzed light emission under a low-light video-imaging camera. Tumor-specific light emission was detected 4 days after viral injection in nude mice carrying subcutaneous C6 glioma implants ranging in size from 25 to 2500 mm³. The signal became more intense after the 4th postinjection day and lasted for 30 to 45 days, indicating continued viral replication. Tumor accumulation of rVV-ruc-gfp virus particles was also seen in nude mice carrying subcutaneous tumors developed from implanted PC-3 human prostate cells, and in mice with orthotopically implanted MCF-7 human breast tumors. Further, intracranial C6 rat glioma cell implants in immunocompetent rats and MB-49 human bladder tumor cell implants in C57 mice were also targeted by the *Vaccinia* virus. Cross sections of a C6 glioma revealed that light emission was clustered in "patches" at the periphery of the tumor where the fast-dividing cells reside. In contrast, cross sections of breast tumors revealed that fluorescent "islands were distributed throughout the tumors. In addition to primary breast tumors, small metastatic tumors were also detected externally in the contralateral breast region, as well as in nodules on the exposed lung surface, suggesting metastasis to the contralateral breast and lung. In summary, light-emitting cells or microorganisms, e.g. *Vaccinia* virus can be used to detect and treat primary and metastatic tumors.

[0007] Similar results were obtained with light-emitting bacteria (*Salmonella*, *Vibrio*, *Listeria*, *E. coli*) which were

injected intravenously into mice and which could be visualized in whole animals under a low light imager immediately. No light emission was detected twenty-four hours after bacterial injection in both athymic (nu/nu) mice and immuno-competent C57 mice as a result of clearing by the immune system. In the cutaneous wound of an intravenously injected animal, the bacterial light emission increases and remains detectable up to six days post-injection. In nude mice bearing tumors developed from implanted C6 glioma cells, light emission was abolished from the animal entirely twenty-four hours after delivery of bacteria, similar to mice without tumors. However, forty-eight hours post-injection, unexpectedly, a strong, rapidly increasing light emission originated only from the tumor regions was observed. This observation indicates a continuous bacterial replication in the tumor tissue. The extent of light emission is dependent on the bacterial strain used. The homing-in process together with the sustained light emission was also demonstrated in nude mice carrying prostate, bladder, and breast tumors. In addition to primary tumors, metastatic tumors could also be visualized as exemplified in the breast tumor model. Tumor-specific light emission was also observed in immunocompetent C57 mice with bladder tumors as well as in Lewis rats with brain glioma implants. Once in the tumor, the light-emitting bacteria were not observed to be released into the circulation and to re-colonize subsequently implanted tumors in the same animal. Further, mammalian cells expressing the Ruc-GFP fusion protein, upon injection into the bloodstream, were also found to home into and propagate in glioma tumors.

[0008] These findings open the way for (a) designing multifunctional viral vectors useful for the detection of tumors based on signals like light emission and/or for suppression of tumor development and/or angiogenesis signaled by, e. g., light extinction and (b) the development of bacterium- and mammalian cell-based tumor targeting systems in combination with therapeutic gene constructs for the treatment of cancer. These systems have the following advantages: (a) They target the tumor specifically without affecting normal tissue; (b) the expression and secretion of the therapeutic gene constructs are, preferably, under the control of an inducible promoter, enabling secretion to be switched on or off; and (c) the location of the delivery system inside the tumor can be verified by direct visualization before activating gene expression and protein delivery.

[0009] Accordingly, the present invention relates to a diagnostic or pharmaceutical composition comprising a micro-organism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal.

[0010] Any microorganism or cell is useful for the diagnostic method of the present invention, provided that they replicate in the organism, are not pathogenic for the organism e.g. attenuated and, are recognized by the immune system of the organism, etc.

[0011] In a preferred embodiment, the diagnostic or pharmaceutical composition comprises a microorganism or cell containing a DNA sequence encoding a luminescent and/or fluorescent protein.

[0012] As used herein, the term "DNA sequence encoding a luminescent and/or fluorescent protein" also comprises a DNA sequence encoding a luminescent and fluorescent protein as fusion protein.

[0013] In an alternative preferred embodiment, the diagnostic or pharmaceutical composition of the present invention comprises a microorganism or cell containing a DNA sequence encoding a protein capable of inducing a signal detectable by magnetic resonance imaging (MRI), e.g. metal binding proteins. Furthermore, the protein can bind contrast agents, chromophores, ligands or compounds required for visualization of tissues.

[0014] Preferably, for transfecting the cells the DNA sequences encoding a luminescent and/or fluorescent protein are present in a vector or an expression vector. A person skilled in the art is familiar with examples thereof. The DNA sequences can also be contained in a recombinant virus containing appropriate expression cassettes. Suitable viruses that may be used in the diagnostic or pharmaceutical composition of the present invention include baculovirus, vaccinia, sindbis virus, Sendai virus, adenovirus, an AAV virus or a parvovirus, such as MVM or H-1. The vector may also be a retrovirus, such as MoMuLV, HaMuSV, MuMTV, RSV or GaLV. For expression in mammals, a suitable promoter is e.g. human cytomegalovirus "immediate early promoter" (pCMV). Furthermore, tissue and/or organ specific promoters are useful. Preferably, the DNA sequences encoding a luminescent and/or fluorescent protein are operatively linked with a promoter allowing high expression. Such promoters, e.g. inducible promoters are well-known to the person skilled in the art.

[0015] For generating the above described DNA sequences and for constructing expression vectors or viruses which contain said DNA sequences, it is possible to use general methods known in the art. These methods include e.g. in vitro recombination techniques, synthetic methods and in vivo recombination methods as described in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, for example. Methods of transfecting cells, of phenotypically selecting transfectants and of expressing the DNA sequences by using the above described vectors are known in the art.

[0016] The person skilled in the art knows DNA sequences encoding luminescent or fluorescent proteins that can be used in the diagnostic or pharmaceutical of the present invention. During the past decade, the identification and isolation of structural genes encoding light-emitting proteins from bacterial luciferase from *Vibrio harveyi* (Belas et al., Science 218 (1982), 791-793) and from *Vibrio fischerii* (Foran and Brown, Nucleic acids Res. 16 (1988), 177), firefly luciferase (de Wet et al., Mol. Cell. Biol. 7 (1987), 725-737), aequorin from *Aequorea victoria* (Prasher et al., Biochem.

26 (1987), 1326-1332), *Renilla* luciferase from *Renilla reniformis* (Lorenz et al., PNAS USA 88 (1991), 4438-4442) and green fluorescent protein from *Aequorea victoria* (Prasher et al., Gene 111 (1987), 229-233) have been described that allow the tracing of bacteria or viruses based on light emission. Transformation and expression of these genes in bacteria allows detection of bacterial colonies with the aid of the low light imaging camera or individual bacteria under the fluorescent microscope (Engebrecht et al., Science 227 (1985), 1345-1347; Legocki et al., PNAS 83 (1986), 9080-9084; Chalfie et al., Science 263 (1994), 802-805).

[0017] Luciferase genes have been expressed in a variety of organisms. Promoter activation based on light emission, using *lux AB* fused to the nitrogenase promoter, was demonstrated in *Rhizobia* residing within the cytoplasm of cells of infected root nodules by low light imaging (Legocki et al., PNAS 83 (1986), 9080-9084; O'Kane et al., J. Plant Mol. Biol. 10 (1988), 387-399). Fusion of the *lux A* and *lux B* genes resulted in a fully functional luciferase protein (Escher et al., PNAS 86 (1989), 6528-6532). This fusion gene (*Fab2*) was introduced into *Bacillus subtilis* and *Bacillus megatherium* under the xylose promoter and then fed into insect larvae and was injected into the hemolymph of worms. Imaging of light emission was conducted using a low light video camera. The movement and localization of pathogenic bacteria in transgenic arabidopsis plants, which carry the pathogen-activated PAL promoter-bacterial luciferase fusion gene construct, was demonstrated by localizing *Pseudomonas* or *Ervinia spp.* infection under the low light imager as well as in tomato plant and stacks of potatoes (Giacomin and Szalay, Plant Sci. 116 (1996), 59-72).

[0018] All of the luciferases expressed in bacteria require exogenously added substrates such as decanal or coelenterazine for light emission. In contrast, while visualization of GFP fluorescence does not require a substrate, an excitation light source is needed. More recently, the gene cluster encoding the bacterial luciferase and the proteins for providing decanal within the cell, which includes *luxCDABE* was isolated from *Xenorhabdus luminescens* (Meighen and Sztitner, J. Bacteriol. 174 (1992), 5371-5381) and *Photobacterium leiognathi* (Lee et al., Eur. J. Biochem. 201 (1991), 161-167) and transferred into bacteria resulting in continuous light emission independent of exogenously added substrate (Fernandez-Pinas and Wolk, Gene 150 (1994), 169-174). Bacteria containing the complete *lux* operon sequence, when injected intraperitoneally, intramuscularly, or intravenously, allowed the visualization and localization of bacteria in live mice indicating that the luciferase light emission can penetrate the tissues and can be detected externally (Contag et al., Mol. Microbiol. 18 (1995), 593-603).

[0019] Preferably, the microorganism is a bacterium, e.g. attenuated *Particularly preferred is attenuated Salmonella typhimurium*, attenuated *Vibrio cholerae* or attenuated *Listeria monocytogenes* or *E.coli*. Alternatively, viruses such as *Vaccinia* virus, AAV, a retrovirus etc. are also useful for the diagnostic and therapeutic compositions of the present invention. Preferably, the virus is *Vaccinia* virus.

[0020] Preferably, the cell of the diagnostic or therapeutic composition of the present invention is a mammalian cell such as stem cells which can be autologous or heterologous concerning the organism.

[0021] In a further preferred embodiment of the diagnostic or therapeutic composition of the present invention the luminescent or fluorescent protein is a luciferase, green fluorescent protein (GFP) or red fluorescent protein (RFP).

[0022] In a particularly preferred embodiment, the microorganism or cell of the diagnostic or pharmaceutical composition of the present invention additionally contains a gene encoding a substrate for the luciferase. In an even more preferred embodiment, the microorganism or cell of the diagnostic or pharmaceutical composition of the present invention contains a *ruc-gfp* expression cassette which contains the *Renilla* luciferase (*ruc*) and *Aequorea gfp* cDNA sequences under the control of a strong synthetic early/late (PE/L) promoter of *Vaccinia* or the *luxCDABE* cassette.

[0023] A preferred use of the microorganisms and cells described above is the preparation of a diagnostic composition for tumor-imaging. The diagnostic composition of the present invention can be used e.g. during surgery, to identify tumors and metastasis. Furthermore, the diagnostic composition of the present invention is useful for monitoring a therapeutic tumor treatment. Suitable devices for analysing the localization or distribution of luminescent and/or fluorescent proteins in an organism, organ or tissue are well known to the person skilled in the art and, furthermore described in the literature cited above as well as the Examples, below. Additionally, the microorganisms and cells can be modified in such a way that they bind metals and consequently are useful in MRI technology to make this more specific.

[0024] The present invention also relates to a pharmaceutical composition containing a microorganism or cell as described above, wherein said microorganism or cell furthermore contains one or more expressible DNA sequence(s) encoding (a) protein(s) suitable for tumor therapy and/or elimination of metastatic tumors, such as a cytotoxic protein, a cytostatic protein, a protein inhibiting angiogenesis, or a protein stimulating apoptosis. Such proteins are well-known to the person skilled in the art. Furthermore, the protein can be an enzyme converting an inactive substance (pro-drug) administered to the organism into an active substance, i.e. toxin, which is killing the tumor or metastasis. For example, the enzyme can be glucuronidase converting the less toxic form of the chemotherapeutic agent glucuronylidoxorubicin into a more toxic form. Preferably, the gene encoding such an enzyme is directed by a promoter which is inducible additionally ensuring that the conversion of the pro-drug into the toxin only occurs in the target tissue, i.e. tumor. Such promoters are e.g. IPTG-, antibiotic-, heat-, pH-, light-, metal-, aerobic-, host cell-, drug-, cell cycle- or tissue specific-inducible promoters. Additional examples of suitable proteins are human endostatin and the chimeric PE37/TGF- α fusion protein. Endostatin is a carboxyterminal peptide of collagen XVIII which has been characterized (Ding et al.,

PNAS USA 95 (1998), 10443). It has been shown that endostatin inhibits endothelial cell proliferation and migration, induces G1 arrest and apoptosis of endothelial cells in vitro, and has antitumor effect in a variety of tumor models. Intravenous or intramuscular injection of viral DNA and cationic liposome-complexed plasmid DNA encoding endostatin result in limited expression levels of endostatin in tumors. However intratumoral injection of purified endostatin shows remarkable inhibition of tumor growth. *Pseudomonas* exotoxin is a bacterial toxin secreted by *Pseudomonas aeruginosa*. PE elicits its cytotoxic effect by inactivating elongation factor 2 (EF-2), which results in blocking of protein synthesis in mammalian cells. Single chain PE is functionally divided into three domains: domain Ia is required for binding to cell surface receptor, domain II is required for translocating the toxin into the target cell cytosol, and domain III is responsible for cytotoxicity by inactivating EF-2. PE40 is derived from wild type *Pseudomonas* exotoxin that lacks the binding domain Ia. Other proteins such as antibody fragments or protein ligands can be inserted in place of the binding domain. This will render the PE40-ligand fusion protein specific to its receptor. One of the highly specific engineered chimeric toxins is the TGF alpha/PE40 fusion protein, where the C-terminus of TGF alpha polypeptide has been fused in frame with the N-terminus of the PE40 protein. TGF alpha is one of the ligands of epidermal growth factor receptor (EGFR), which has been shown to be preferentially expressed on the surface of a variety of tumor cells. TGF alpha-PE40 fusion protein has been shown to be highly toxic to tumor cells with elevated EGFRs on the cell surface and while it is less toxic to nearby cells displaying fewer numbers of surface EGFR. The toxicity of TGF alpha-PE40 chimeric protein is dependent on a proteolytic processing step to convert the chimeric protein into its active form, which is carried out by the target. To overcome the requirement for proteolysis, a new chimeric toxin protein that does not require processing has been constructed by Theuer and coworkers (J.Biol.Chem. 267 (1992), 16872). The novel fusion protein is termed PE37/TGF alpha, which exhibited higher toxicity to tumor cells than the TGF alpha-PE40 fusion protein.

[0025] Thus, in a preferred embodiment of the pharmaceutical composition, the protein suitable for tumor therapy is endostatin (for inhibition of tumor growth) or recombinant chimeric toxin PE37/transforming growth factor alpha (TGF-alpha) (for cytotoxicity to tumor cells).

[0026] Moreover, the delivery system of the present application even allows the application of compounds which could so far not be used for tumor therapy due to their high toxicity when systemically applied. Such compounds include proteins inhibiting elongation factors, proteins binding to ribosomal subunits, proteins modifying nucleotides, nucleases, proteases or cytokines (e.g. IL-2, IL-12 etc.), since experimental data suggest that the local release of cytokines might have a positive effect on the immunosuppressive status of the tumor.

[0027] Furthermore, the microorganism or cell can contain a BAC (Bacterial Artificial Chromosome) or MAC (Mammalian Artificial Chromosome) encoding several or all proteins of a specific pathway, e.g. anti-angiogenesis, apoptosis, woundhealing-pathway or anti-tumor growth. Additionally the cell can be cyber cell or cyber virus encoding these proteins.

[0028] For administration, the microorganisms or cells of the present invention are preferably combined with suitable pharmaceutical carriers. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc.. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the microorganisms or cells may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The preferred route of administration is intravenous injection. The route of administration, of course, depends on the nature of the tumor and the kind of microorganisms or cells contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind, size and localization of the tumor, general health and other drugs being administered concurrently.

[0029] Preferred tumors that can be treated with the microorganisms or cells of the present invention are bladder tumors, breast tumors, prostate tumors, glioma tumors, adenocarcinomas, ovarian carcinomas, and pancreatic carcinomas; liver tumors, skin tumors.

Brief description of the drawings

[0030]

Figure 1: External imaging of GFP expression in subcutaneous C6 glioma tumors in nude mice

C6 glioma cells (5×10^5) were implanted subcutaneously into the right lateral thigh. At designated days after tumor cell implantation, the animals were infected intravenously with 1×10^8 pfu of rVV-ruc-gfp virus particles. GFP expression was monitored under a fluorescence stereomicroscope. Bright field (top), fluorescence (middle), and bright field, fluorescence overlay (bottom) images of subcutaneous glioma tumor are shown. GFP signal can be observed in tumors as small as 22 mm³ in size (B-B'), or as old as 18 days (about 2500 mm³ in size) (A-A'). In

older tumors, GFP expression was seen in "patch"-like patterns (indicated by arrows in A'). Marker gene expression in the tumor of the same animal can be monitored continuously 4 (C-C'), 7 (D-D'), and 14 (E-E') days after intravenous viral injection. (Bars = 5 mm.)

Figure 2: Visualization of tumor angiogenesis

C6 glioma cells (5×10^5) were implanted subcutaneously into the right lateral thigh of nude mice. Ten days after tumor cell implantation, the animals were infected intravenously with 1×10^8 pfu of rVV-ruc-gfp. GFP expression was monitored 7 days post-viral injection. Vascularization at the surface of the subcutaneous C6 glioma tumor is shown against the bright green fluorescent background in the tumor following *Vaccinia*-mediated gene expressions. Bright field (A), fluorescence (B), and bright field, fluorescence overlay (C) images of subcutaneous glioma tumor are illustrated. (Bars = 5 mm.)

Figure 3: Expression of GFP in subcutaneous glioma tumor of the same animal

Five days after the subcutaneous implantation of 5×10^5 C6 glioma cells into the right lateral thigh, 10^8 of rVV-ruc-gfp virus particles were injected intravenously. Five days after viral injection, the animal was anesthetized and sacrificed for analysis of GFP expression under fluorescence microscope. The tumor was visualized externally (A-K), with the overlying skin reflected (B-B'), in cross section (C-C'), and in the amputated leg (D-D'). Bright field (A), fluorescence (B), and bright field, fluorescence overlay (C) images of subcutaneous glioma tumor are illustrated. The strongest GFP expressions are seen as patches located along the outer surface of the tumor on the right (double arrows in C-C'). Sharp difference of GFP expression in tumor tissue and in the normal muscle tissue (arrows in D-D') is clearly visible. Asterisks mark the reflected skin (B-B' and D-D'). (Bars = 5 mm.)

Figure 4: Bright field (A) and fluorescence (B) images of tumor cells expressing GFP

Frozen sections ($30 \mu\text{m}$ thick) of the glioma tumor tissues were prepared from a nude mouse that has been intravenously injected with 1×10^8 of rVV-rucgfp virus particles. (Bars = $50 \mu\text{m}$.)

Figure 5: Low light image of the anesthetized nude mouse to indicate the location of Renilla luciferase-triggered light emission in the presence of intravenously injected substrate coelenterazine ($5 \mu\text{g}$ ethanol solution)

Figure 6: Monitoring tumor-specific viral infection based on GFP gene expression in a variety of tumor models including subcutaneous PC-3 human prostate tumor (A-A') and MCF-7 human breast tumor (B-B') in nude mice, intracranial C6 rat glioma tumor (C-C', arrows indicate the location of the tumor) in Lewis rats, and MB-49 human bladder tumor (D-D') in C57 mice. Animals were monitored 7 days after intravenous injections of 1×10^8 of rVV-ruc-gfp virus particles. Bright field (top), fluorescence (middle), and bright field, fluorescence overlay (bottom) images of the tumor are illustrated. (Bars = 5 mm.)

Figure 7: Monitoring Vaccinia-mediated GFP expression in a breast tumor model

Nude mouse carrying breast tumor was injected intravenously with 1×10^8 of rVV-ruc-gfp virus particles. Both the primary tumor (A-A', B-B', and C-C') and the metastasized tumor (D-D', E-E', and F-F') were visualized externally (A-A' and D-D'), with overlying skin removed (B-B' and E-E'), and when they were split open (C-C' and F-F') in a set of bright field, fluorescence (*) and bright field, fluorescence overlay (") images. GFP expression in lung metastases in the same animal was also visualized (G-G'). (Bars = 5 mm (A-A' to F-F'), and Bars = 1 mm (G-G').

Figure 8: Visualization of the clearance of light emitting bacteria from nude mice based on the detection of light emission under the low light imager

Nude mice were intravenously injected with 10^7 cells of attenuated *S. typhimurium* (A, B) and *V. cholera* (C, D). Both strains were transformed with pLITE201 carrying the *lux* operon. Photon collection was done 20 min (A, C) and 2 days (B, D) after bacterial injections.

Figure 9: Homing of glioma tumors by attenuated bacteria

Nude mice with a C6 glioma tumor in the right hind leg were intravenously injected with 10^7 attenuated *S. typhimurium* (A-D) and with *V. cholera* (E-H) both transformed with pLITE201 plasmid DNA encoding the *lux* operon. Photon collection was carried out for one minute under the low light imager. Mice injected with *S. typhimurium* exhibited luminescence immediately through the whole animal (A). In contrast, luminescence in the mice injected with *V. cholera* was visible in the liver area (E). Two days after bacterial injection, both groups of mice demonstrated luminescence only in the tumor region (B, F). The light emission in the tumors infected with *S. typhimurium* slowly diminished four (C) and six (D) days after bacterial injection. Tumors infected with *V. cholera* showed enormously increased light emission four (G) and six (H) days after injection suggesting continued replication of the bacteria

in the tumor tissues.

Figure 10: Homing in of bacteria onto breast tumors

Nude mice with breast tumors in the right breast pad were intravenously injected with 10^7 attenuated *V. cholera* (A-D) and with 10^7 *E. coli* (E-F) transformed with pLITE201 plasmid DNA encoding the *lux* operon. Photon collection was carried out for one minute under the low light imager. Twenty minutes after bacterial delivery, luminescent *V. cholera* were observed in the liver (A). Forty-eight hours after injection, light emission was noted in the primary breast tumor in the right breast area and a metastatic tumor (arrow) in the left breast area, and in the incision wound (B). At five days, the light emission was visible only in the tumor regions, and non at the wound (C). Eight days after bacterial injection, the luminescent activity was abolished from the smaller tumor region but remained strong in the primary breast tumor (D). Homing in of *E. coli* onto breast tumors in nude mice was also observed two days after intravenous bacterial injection (E: side view, F: ventral view).

Figure 11: Homing in of bacteria onto bladder tumors in C57 mice

C57 mice were intravenously injected with 10^7 attenuated *V. cholera* transformed with pLITE201 encoding the *lux* operon. Nine days after bacterial delivery, luminescence was noted in the bladder region of the whole animal (A). The animal was sacrificed and an abdominal incision was made to expose the bladder. The light emission was limited to the bladder region (B). With the removal of the bladder (C) from the mouse, the entire source of light emission was removed (D) as demonstrated by the overlay of the low light photon emission image over the photographic image of the excised bladder.

Figure 12: Homing in of bacteria onto brain glioma tumors in Lewis rats

Lewis rats were intravenously injected with 10^8 cells of attenuated *V. cholera* transformed with pLITE201 encoding the *lux* operon. Twenty-four hours after bacterial injection, faint luminescence was noted in the head region of the whole animal during visualization under the low light imager. The animals were sacrificed and their brain removed. Photon collection was carried out for one minute from rats with (A) and without (B) brain tumors. Strong luminescence was confirmed in regions of the brain of the rats with the brain tumor (marked with arrows in A). Luminescence was completely absent in the control brain tissues (B).

Figure 13: Transformed human fibrosarcoma cells home in on subcutaneous glioma tumors in nude mice

Nude mice with human breast tumors were injected intravenously with 5×10^5 human fibrosarcoma cells, which were permanently transformed with retrovirus derived from pLEIN. Seven days post-injection, the animals were anesthetized and monitored under a fluorescent stereomicroscope. Fluorescent cells were noted only in the tumor region of the whole mice through the skin (A1-3). Upon exposure of the tumor tissues by reflection of the overlying skin (B1-3), and in cross sections of the tumors (C1-3), fluorescent patches were visible in distinct regions. Close examination of the organs of the mice showed the presence of small clusters of fluorescent cells in the lungs of the animals, demonstrating the affinity of the fibrosarcoma cells for the lungs in addition to the tumorous tissues (D1-3). (Bars = 5 mm (A1-C3), = 1 mm (D1-D3)).

Figure 14: Homing of attenuated *Listeria monocytogenes* into subcutaneous prostate tumors

Nude mice with subcutaneous human PC3 prostate tumor in the right hind leg were intravenously injected with 10^7 attenuated *L. monocytogenes* transformed with pSOD-gfp plasmid DNA carrying the gfp cDNA. GFP fluorescence was observed under a fluorescence stereo microscope. Twenty-seven hours after bacterial injection, GFP signal was detected only in the tumor region. The tumor is shown in a set of visible light (a), fluorescent (b), and visible and fluorescent light overlay (C) images. (Bars = 5 mm.)

[0031] The present invention is explained by the examples.

Example 1: Materials and Methods

[0032]

(A) Bacterium strains. The bacterial strains used were attenuated *Salmonella typhimurium* (SL7207 hisG46, DEL407[aroA544::Tn10]), attenuated *Vibrio cholerae* (Bengal 2 Serotyp O139, M010 DattRS1) and attenuated *Listeria monocytogenes* (D2 mpl, actA, plcB). The bacterial strains were kindly provided by Prof. W. Göbel (University of Würzburg, Germany).

(B) Plasmid constructs. The plasmid pLITE201 containing the *luxCDABE* cassette was obtained from (Voisey

and Marincs, Biotech 24,1998, 56-58). The plasmid pXyIA-dual with the operon sequence of *gfp*-cDNA, *lux* AB, *lux* CD, and *lux* E under the control of the Xylose promoter was kindly provided by Dr. Phil Hill (University of Nottingham, UK).

5 **(C) Transformation of bacteria**

The bacteria were transformed by electroporation.

10 **(D) Tumor Cell lines.** The rat C6 nitrosourea-induced glioma cell line (ATCC, Rockville, MD) was cultured in RPMI-1640 medium (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 10% (v/v) FBS and 1 x penicillin/streptomycin. The human PC3 prostate carcinoma cell line (ATCC, Rockville, MD) and the Human MB-49 bladder tumor cells and rat 9L glioma cells were maintained in DMEM medium (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with L-glutamine and 10% (v/v) FBS. HT1080 fibrosarcoma cells (ATCC, Manassas, VA) were cultured in F12 minimal essential media (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 10% FBS and 1 x penicillin/streptomycin. The MCF-7 human mammary carcinoma cell line (ATCC, Rockville, MD), permanently transformed with a plasmid carrying pro-IGF-II cDNA (obtained from Dr. Daisy De Leon, Loma Linda University, Loma Linda, CA) was cultured in DMEM/F12 medium supplemented with 5% FBS and 560 µg/ml of G418 (Life Technologies, Grand Island, NY).

20 **(E) Production and propagation of retrovirus to generate a light-emitting stably transformed cell line.** PT67 packing cells (Clontech, Palo Alto, CA) were cultured in DMEM medium supplemented with 10% (v/v) FBS. At 70% confluence, PT67 cells were transformed with pLEIN (Clontech, Palo Alto, CA) using calcium phosphate precipitation method (Profection Mammalian Transfection Systems, Promega, Madison, WI) for 12 hours. Fresh medium was replenished at this time. Retroviral supernatant collected from PT67 cells 48 hours post transformation were filtered through a 0.45 µm filter and was added to target HT1080 cells along with polybrene to a final concentration of 4 µg/ml. The medium was replaced after 24 hours and the cells were treated with G418 selection at 400 µg/ml and stepwise increased to 1200 µg/ml.

30 **(F) Recipient animals and tumor models.** Five- to six-week-old male BALB/c athymic nu/nu mice (25-30 g in body weight) and Lewis rats (250-300 g in body weight) were purchased from Harlan (Frederick, MD). C57BL/6J *Min*⁺ mice were obtained from Jackson Laboratories (Bar Harbor, ME). *Min* (multiple intestinal neoplasia) is an autosomal dominant trait involving a nonsense mutation in codon 850 of the murine *Apc* gene, which renders these animals susceptible to spontaneous intestinal adenoma formation. Female BALB/c athymic nu/nu mice bearing MCF-7 human breast tumor implants were generated and kindly provided by Dr. Daisy DeLeon and Dr. Tian (Loma Linda University, Loma Linda, CA). C57 mice with orthotopically implanted human MB-49 tumor cells in the bladder were generated and kindly provided by Dr. Istvan Fodor (Loma Linda University, Loma Linda, CA). All animal experiments were carried out in accordance with protocol approved by the Loma Linda University animal research committee. The animals containing recombinant DNA materials and attenuated pathogens were kept in Loma Linda University animal care facility under biosafety level two.

40 **(G) Propagation of recombinant Vaccinia virus.** *Vaccinia* virus Lister strain (LIVP) was used as a wild type virus. Recombinant *Vaccinia* virus rVV-ruc-gfp was constructed by inserting, via homologous recombination, the ruc-gfp-cassette into the *Vaccinia* virus genome (Wang et al., Proc. Biolumin. Chemilumin. 9, 1996, 419-422). The virus was amplified in CV-1 cells by addition of virus particles at a multiplicity of infection (MOI) of 0.1 pfu/cell to CV-1 cell monolayers followed by incubation at 37°C for 1 h with brief agitation every 10 min. At this time, the supernatant fluid with virus particles was removed, and the cell monolayers were washed once with serum free medium. Complete growth medium was then added and the cells were incubated at 37°C. rVV-ruc-gfp virions propagated in CV-1 cells were purified through a sucrose gradient. A plaque assay was used 72 h after infection to determine the titer of recombinant virus by staining the cells with 50% crystal violet solution in ethanol.

50 **(H) Generation of mice carrying tumor implants.** To obtain tumors in nude mice, C6 glioma cells were grown, harvested and the cell number was determined by the Trypan Blue exclusion method. Disinfectant was applied to the skin surface, then approximately 5×10^5 cells were suspended in 100 µl of phosphate buffered saline (PBS) and injected subcutaneously into the right lateral thigh of each mouse. Tumor growth was monitored by recording the size of the tumor with a digital caliper. Tumor volume (mm³) was estimated by the formula $(L \times H \times W)/2$, where L is the length, W is the width, and H is the height of the tumor in mm.

55 Intracerebral glioma tumors were generated by injecting C6 glioma cells into the head of rats. Prior to injection, rats were anesthetized with sodium pentobarbital (Nembutal® Sodium solution, Abbot Laboratories, North Chicago, IL; 60 mg/kg body weight). A midline scalp incision (0.5 - 1 cm) was made, the skin was retracted, and a 1 mm

EP 1 281 767 A2

burr hole was made in the skull at a location 2 mm to the left and 2.5 mm posterior to the bregma. Tumor cells were pipetted into an insulin syringe, which was fitted with a 29-gauge needle and mounted in a stereotactic holder. The needle was inserted vertically through the burr hole to a depth of 3 mm. After injection into the brain of 5×10^5 C6 cells in a 10 μ l volume, the needle was kept in place for 15 sec and then withdrawn. The skin incision was closed with surgical clips. Mice bearing subcutaneous prostate tumors were generated over a period of one month following subcutaneous implantation of 3×10^6 PC3 human prostate cells.

MB-49 human bladder tumor cells were implanted in the C57 mouse bladder to produce animals with bladder tumors. To generate animals with breast cancer (Tian and DeLeon, submitted for publication), female nude mice were first implanted with 0.72 mg/90 day-release 17β -estradiol pellets (Innovative Research, Rockville, MD) in the skin to facilitate breast tumor development and metastasis. One day after estrogen pellet implantation, 1×10^6 MCF-7 human breast carcinoma cells transformed with pro-IGF-II (Dull et al., Nature 310 (1984), 777-781) were implanted in the mammary fat pad. For orthotopic transplants, tumors developed from implanted cells were resected and minced into 1-mm³ cubes for tissue transplantation into the mammary fat pad.

(I) Assay of *Renilla* luciferase in live animals. Mice were anesthetized with Nembutal (60 mg/kg body weight) before every *Renilla* luciferase assay. *Renilla* luciferase activities were determined after intravenous injection of a mixture of 5 μ l of coelenterazine (0.5 μ g/ μ l diluted ethanol solution) and 95 μ l of luciferase assay buffer (0.5 M NaCl; 1 mM EDTA; and 0.1 M potassium phosphate, pH 7.4). Whole live animals were then imaged in a dark box using a Hamamatsu low light video camera, and the images were recorded using Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, MD). The pseudocolored photon emission image was superimposed onto the gray scale image of the animal in order to precisely locate the site of light emission.

(J) Fluorescence microscopy of live animals. Mice were anesthetized with Nembutal (60 mg/kg body weight) before tumor visualization. External imaging of GFP expression in live animals was performed using a Leica MZ8 stereo fluorescence microscope equipped with a mercury lamp power supply and a GFP filter (excitation at 470 nm). Images were captured using a SONY DKC-5000 3CCD digital photo camera.

(K) Detection of luminescence and fluorescence. Immediately before imaging, mice and rats were anesthetized with Nembutal® (60 mg/kg body weight). The animals were placed inside the dark box for photon counting and recording superimposed images (ARGUS 100, Hamamatsu, Hamamatsu, Japan). Photon collection was for one minute from ventral and dorsal views of the animals. A light image was then recorded and the low light image was then superimposed over the light image to record the location of luminescent activity.

Imaging of GFP expression in tumors of live animals was performed using a Leica MZ8 stereo fluorescence microscope equipped with a mercury lamp power supply and a GFP filter (excitation at 470 nm). Images were captured using a SONY DKC-5000 3CCD digital photo camera.

(L) Histology of tumor tissues. Under anesthesia, the animals were euthanized with an overdose of Nembutal®. The tissues of interest were removed, embedded in Tissue-Tek OCT compound (Miles Scientific, Naperville, IL) and immediately frozen in liquid nitrogen without fixation. Frozen sections were cut at -20°C using a Reichert-Jung Cryocut 1800 cryostat. GFP fluorescence of the tissues was monitored under a Leica fluorescence microscope and the images were recorded using Photoshop software.

Example 2: Results obtained by intravenous injection of recombinant *Vaccinia* virus rVV-ruc-gfp into mice

(A) Monitoring of virus-mediated marker gene expression in immunodeficient mice

[0033] *Vaccinia* virus (1×10^8 pfu) carrying the *Renilla* luciferase - GFP fusion expression cassette (rVV-ruc-gfp) was introduced intravenously into nude mice with no tumors. The animals were observed once every 3 days over a two-week time period under the low-light imager to monitor luciferase catalyzed light emission immediately after intravenous injection of coelenterazine, and under a fluorescence microscope to visualize GFP expression. Neither apparent luminescence nor green fluorescence was detected in the animals when imaged externally, except at certain locations that had small skin lesions. Such luminescence and fluorescence signals disappeared after a few days once the lesions had healed. Animals were sacrificed one week and two weeks after viral infection, and their organs were removed and examined for the presence of luminescence and GFP fluorescence signals. One week after viral injection, no luminescence or green fluorescence could be detected in brain, liver, lung, spleen, kidney or testis. These results indicated that the rVV-ruc-gfp virus did not show organ specificity after injection and that the virus seemed to be cleared from the animal by the immune system soon after systemic delivery via the bloodstream.

(B) Visualization of Vaccinia virus-mediated marker gene expression in glioma tumors of live nude mice

[0034] The distribution of injected *Vaccinia* virus in nude mice bearing subcutaneously implanted C6 glioma tumors was examined. Nude mice with tumors approximately 500 mm³ in size were injected intravenously with 1 x 10⁸ pfu of the rVV-ruc-gfp virus. Seven days after virus injection, the animals were monitored for GFP expression under a fluorescence microscope to determine the presence of viral infection and multiplication in the tumors, which had grown to approximately 2500 mm³ in size. Surprisingly, green fluorescence was detected only in the tumor regions in live animals. Seven days after viral injection, the GFP fluorescence was very intensely localized in a patch-like pattern restricted to the tumor region (Fig. 1A-A"). These patches, often seen at the end of blood vessel branches, may have indicated local viral infection of tumor cells that surround the leaky terminals of capillary vessels. During real-time observation of the same tumors, the GFP signal from the center of these patches started to disappear, and new green fluorescent centers appeared in the form of rings at the periphery of the fading patches. The new sites of intense GFP fluorescence may have resulted from progression of the viral infection to nearby cells within the tumor during tumor growth and expansion. After careful examination of the mice, with the exception of the tumor region, no detectable green fluorescence was seen elsewhere on the body surface or in the dissected organs. This experiment clearly showed that a mature solid tumor could be easily localized by the labeled *Vaccinia* virus, based on light-emission, and it also demonstrated the affinity of virus particles for the tumor tissue.

[0035] To determine whether tumor size and vascularization are decisive factors for viral retention in tumors, nude mice were intravenously injected with 1 x 10⁸ rVV-ruc-gfp *Vaccinia* virus particles one day after subcutaneous C6 cell implantation. Surprisingly, 4 days after viral injection GFP expression was seen in 5-day-old C6 tumors that had a volume of about 25 mm³ (Fig. 1B-B"). Examination of labeled *Vaccinia* virus tumor targeting by visualization of GFP expression in implanted tumors younger than 5 days was not feasible in live mice, since sufficient levels of marker gene expression required approximately 4 days to allow detection under a fluorescence microscope.

[0036] The finding that injection of the rVV-ruc-gfp *Vaccinia* virus into the bloodstream of the host resulted in GFP expression and accumulation in tumors suitable for non-invasive tumor detection allowed us to follow the entry and replication process of this virus in the same animal in real time (Fig. 1 C-C", D-D" and E-E"). A continuously increasing level of GFP fluorescence was observed in the same animal throughout 20 days following viral injection, which was the time scheduled before sacrificing the animals. Such an increase in detectable fluorescence was indicative of a very strong viral replication in the tumor tissue, the latter appearing to function as a protective immunoprivileged environment for viral replication. Interestingly, the location of blood vessels and the neovascularization within the periphery of the enlarging tumor were readily visible and confirmed by external visualization against a bright green fluorescent background (Fig. 1A-A", D-D", E-E" and Fig. 2).

[0037] To determine the location of viral infection within the tumors, the animals were sacrificed and the skin over the tumor was carefully reflected to expose the tumor. In the exposed tumor, GFP fluorescence was found to be concentrated exclusively in the tumor tissue (Fig. 3B-B" and D-D"). The non-tumorous thigh muscles did not show any fluorescence of viral infection, as indicated by arrows in Fig. 3D-D". The skin overlying the tumor was also non-fluorescent (indicated by asterisks in Fig. 3B-B" and D-D"). Cross sections of the tumor, however, revealed that strong green fluorescent regions were mostly found as patches in the periphery of the tumor (double arrows in Fig. 3 C-C") where the actively dividing tumor cells are presumably located.

[0038] To further examine the pattern of viral infection in C6 glioma tumors based on GFP expression, the tumor tissues were sectioned for microscopic analysis under the fluorescence microscope. Comparative analysis of various tissue sections revealed that GFP fluorescence was present in large clusters of cells within the tumor (Fig. 4), but no fluorescence was visible in normal tissues such as the heart, lung, liver, spleen, and kidney.

[0039] In addition to GFP, the recombinant rVV-ruc-gfp virus carried a second marker gene, which encoded the Renilla luciferase in the form of a fusion protein with GFP. Therefore we were able to directly superimpose the site of GFP fluorescence with light emission from *Renilla* luciferase in the tumors. Immediately after coelenterazine (substrate for *Renilla* luciferase) was delivered by intravenous injection, a very strong luciferase activity was recorded only in the tumor region under a low light video camera (Fig. 5). By lowering the sensitivity of the low light video camera to avoid saturation of light detection, we were able to identify the *Renilla* luciferase gene expression in localized patches in the periphery of the tumor. These patch-like patterns precisely correlated with the GFP signals.

(C) Affinity of Vaccinia virus delivered to the bloodstream for different tumors implanted into animals

[0040] To determine whether the attraction of the *Vaccinia* virus was limited to glioma tumors or whether this attraction could be observed in other tumors, recombinant *Vaccinia* virus was recombinantly introduced into mice that carried different types of implanted tumors. One of these tumor models was a nude mouse with implanted subcutaneous PC-3 human prostate carcinoma. Although the PC3 implants from which tumors developed grew at a much slower rate than the implanted subcutaneous glioma tumors, these tumors showed the same dynamics with regards to *Vaccinia*

virus infection when identical titers (1×10^8) were injected intravenously (Fig. 6A-A"). Similar to our findings with glioma tumors, GFP expression was initially detected 4 days after virus injection, and the fluorescence lasted throughout the 3-week observation period.

[0041] Female nude mice with established breast tumors were also used for labeled *Vaccinia* injections. These breast tumors were allowed to grow for 6 months after the animals received implants of MCF-7 human breast carcinoma cells transformed with pro-IGF-11 cDNA. At the time of *Vaccinia* virus injection, the tumors had reached maximum growth and the tumor volume (about 400-500 mm³) did not change significantly during the experimental period. Similar to previous experiments, 6 days after intravenous delivery of 1×10^8 rVV-ruc-gfp virus particles, strong GFP expression was observed in the breast tumor region (Fig. 6B-B", Fig. 7A-A" and B-B") and nowhere else in the body.

[0042] Examination of cross sections of virus-infected breast tumors revealed luminescent "islands" throughout the tumors without any indication of central or peripheral preference of infection (Fig. 7C-C"). The MCF-7 tumor cells used in these breast tumor models are known to metastasize and in addition to the primary solid tumor, a smaller metastasized tumor found on the left lateral side of the body showed GFP fluorescence (Fig. 7D-D", E-E", and F-F"). Excised lung tissues were also examined for detection of metastases. Metastasized tumors as small as 0.5 mm in diameter on the surface of the lung were positive for GFP fluorescence (Fig. 7G-G"). The presence of a strong *Renilla* luciferase-mediated light emission confirmed the expression of the luciferase-GFP fusion protein in these breast tumors but nowhere else in the body when the substrate coelenterazine was injected intravenously into the live animals. These experiments showed that intravenously delivered *Vaccinia* virus particles were selectively attracted to and replicated in primary and metastasized breast tumors in nude mice, likely as a result of the immunocompromised state of the tumor microenvironment.

[0043] To determine whether virus particles could move out of tumors and re-enter the circulation, we injected C6 glioma cells into the thigh of mice to form a second tumor in animals already carrying a breast tumor infected with labeled *Vaccinia* virus. If the virus particles were released from the tumor to re-enter the circulation in significant numbers they would be able to colonize the newly implanted glioma tumor. Monitoring of these second tumors showed that no GFP signal was visible in the new glioma tumor 7 and 14 days after implantation of the glioma cells. To demonstrate that the newly implanted glioma tumors could be targeted by labeled *Vaccinia* virus, a second dose of rVV-ruc-gfp virus (1×10^8 pfu) was intravenously injected. Five days later, tumor-specific GFP expression was detected in the newly formed glioma tumor in addition to GFP expression seen in the original breast tumor. These findings suggested that the virus particles in infected tumors were either not released back into the circulation at all, or were not released in sufficient numbers to infect and replicate in a second tumor.

[0044] Two additional tumor models, including Lewis rats with intracranial C6 rat glioma tumors and C57 mice with MB-49 human bladder tumors in the bladder, were used for *Vaccinia* injections. To determine whether tumor-affinity of virus particles is a phenomenon limited to tumors in nude mice with a diminished T-lymphocyte function or whether it is a general protective property of tumors that may be demonstrated also in immunocompetent animals, Lewis rats with intracranial C6 rat glioma tumors and C57 mice with MB-49 human bladder tumors in the bladder were used. A total of 5×10^5 C6 glioma cells in a 100 μ l volume were stereotactically implanted in the brains of 2 of 4 immunocompetent Lewis rats, and the tumors were allowed to grow for 5 days. The other 2 rats were injected intracranially with phosphate-buffered saline to serve as controls. On day six, all 4 rats were intravenously injected with rVV-ruc-gfp virus particles via the femoral vein. Five days after virus injection, all 4 animals were sacrificed, and their brains were carefully excised for analysis by fluorescence microscopy. GFP expression was detected in the brains with implanted intracranial tumors (Fig. 6C-C") while no GFP expression was seen in the control brains. In parallel experiments, C57 mice, with or without bladder tumors, were divided into two groups. One group was injected intravenously with rVV-ruc-gfp *Vaccinia* virus (1×10^8 pfu) and the other with saline solution as control. Five days after virus injection, the animals were sacrificed and examined under the fluorescence microscope. GFP expression was observed in the bladder tumor region in C57 mice but not in control mice (Fig. 6D-D").

[0045] Taken together, these experiments show that *Vaccinia* virus particles were selectively accumulated and retained in a variety of tumors, probably protected by the tumor microenvironment, and that they were not able to survive in the non-tumorous tissues of immunocompromised as well as immunocompetent animals. The tumor-targeting process by intravenously injected *Vaccinia* virus carrying the light-emitting dual marker gene demonstrated the ability of the *Vaccinia* virus system to detect primary and metastatic tumors in live animals.

Example 3: Results of Intravenous Injection of bacterial and mammalian light-emitting cells into mice

(A) Visualization of light emitting bacteria present in whole animals after intravenous injection

[0046] To determine the fate of intravenously injected luminescent bacteria in the animals, 10^7 bacteria carrying the pLITE201 plasmid in 50 μ l were injected into the left femoral vein under anesthesia. Following closure of the incision with sutures, the mice were monitored under the low light imager (ARGUS 100 Camera System, Hamamatsu, Hama-

matsu, Japan) in real time and photons were collected for one minute. The imaging was repeated in two-day time intervals to determine the presence of light emission from a given animal. It was found that the distribution pattern of light emission following an intravenous injection of bacteria into mice was characteristic of the bacterial strains used. Injection of the attenuated *V. cholera* into the bloodstream resulted in light emission localized in the liver immediately. Injection of *S. typhimurium*, however, was widely disseminated throughout the body of the animal suggesting a difference in the interaction with host cell system (Figure 8A-8D). Imaging the same animals 24 and 48 hours post-infection showed that all of the detectable light emission from the earlier time diminished rapidly and was eliminated completely from the injected animal. These findings suggest that light emitting bacteria injected into the bloodstream via the femoral vein are cleared. This process was confirmed by photon emission analysis of excised organs, which were found to lack light emission. Similar data were obtained in immunocompetent mice and rats suggesting that the removal of bacteria from the blood is efficient in both systems.

(B) Bacteria home in on glioma tumors in nude mice

[0047] To determine if bacteria preferentially colonize tumorous tissues, nude mice with ten-day-old tumors (about 500 mm³) in the right hind leg were injected intravenously via the femoral vein with 10⁷ *S. typhimurium* or 10⁷ *V. cholera* in a 50 µl volume of bacterial suspension. Following injection, the incision wounds were sutured and the animals were monitored for six days under the low light imager. At each observation time point, photons were collected for exactly one minute. In mice injected with *S. typhimurium*, luminescent bacteria were disseminated throughout the whole body of the animal similar to the findings in the non-tumorous mice (Figure 9A). Nude mice injected with *V. cholera*, demonstrated luminescent activity only in the liver region during the early observation period (Figure 9E). Regardless of the bacterial strain injected, two days after injection, luminescent activity was observed only in the tumor region (Figures 9B and 9F). Monitoring of the mice under the low light imager on days four and six post-injection showed decreased amounts of detectable luminescence in the tumors of animals injected with *S. typhimurium* (Figures 9C and 9D). This finding was in marked contrast with the findings in the tumors of mice injected with *V. cholera*, which demonstrated not only survival but also propagation of the bacteria in the tumor mass with a dramatic increase in light emission (Figures 9G and 9H).

[0048] Nude mice bearing subcutaneous human PC3 prostate tumors in the right hind leg were intravenously injected with 10⁷ attenuated *L. monocytogenes* transformed with pSOD-gfp plasmid DNA carrying the gfp cDNA. GFP fluorescence was observed under a fluorescence stereomicroscope. Twenty-seven hours after bacterial injection, GFP signal was detected only in the tumor region (Figure 10). No GFP signal was observed in the rest of the animal.

(C) Determination of minimum size and age of glioma tumors necessary for bacterial infection.

[0049] The purpose of this experiment was to determine whether the size of the tumor has any influence on its ability to be colonized by bacteria. Tumors were induced in the right hind leg of nude mice by subcutaneous injection of glioma cells as described. On days 0, 2, 4, 6, 8, and 10 of tumor induction, attenuated *S. typhimurium* and *V. cholera* with the pLITE201 plasmid were injected intravenously through the femoral vein. Presence of luminescent bacteria in the tumor was determined by photon collection for exactly one minute under the low light imager two and four days post-infection. The tumor volume was also determined by measuring the dimensions with a digital caliper. The earliest time-point when luminescent activity was noted in the tumors was on day eight after tumor induction. Corresponding tumor volumes were approximately 200 mm³.

(D) Bacteria home in on breast tumors of nude mice

[0050] In order to determine whether colonization of tumors is limited to glioma cells or whether this is a general phenomenon observed with all tumors, female nude mice bearing tumors in the right breast pad were intravenously injected with 10⁷ *V. cholera* in a 50 µl volume of bacteria suspension. The animals were monitored within the first 10 minutes after inoculation under the low light imager for one minute and demonstrated the typical luminescent pattern in the liver region (Figure 11A). Two days later, while the liver had become clear of luminescent bacteria, the breast tumor was colonized by the labeled *V. cholera*. In addition to the main tumor, a metastatic tumor in the left breast demonstrated luminescent activity (Figure 11B). On day five, the animals had cleared the bacteria that colonized the incision wound, however, the tumors remained luminescent (Figure 11C). Figure 11D shows the continued colonization and propagation of the bacteria in the main tumor, while the metastatic, smaller tumor had become cleared. Luminescent activity continued for over 45 days in the right breast tumor. Similar experiments were conducted using *E. coli* to demonstrate that homing in of tumors by bacteria is not strain dependent (Figures 11E and 11F).

[0051] To determine whether the bacteria from the tumor enter the blood circulation in significant quantities to colonize other sites, a second tumor (C6 glioma) was induced in these animals in the right hind leg. The tumor was allowed to

EP 1 281 767 A2

grow for 10 days. No luminescent activity was observed in the glioma tumor demonstrating the absence of a significant bacteria that would cause colonization of this tumor. However, when the animal was rechallenged with 10^7 attenuated *V. cholera* intravenously, the leg tumor showed strong luminescent activity.

5 [0052] The findings of these experiments demonstrate that larger tumors retain bacteria more effectively over time. Furthermore, the bacteria within the tumors do not escape into the blood in sufficient quantities to infect susceptible sites such as other tumors.

(E) Bacteria home in on bladder tumors in immunocompetent mice

10 [0053] C57 mice were intravenously injected with 10^7 attenuated *V. cholera* transformed with pLITE201 encoding the *lux* operon. On day nine after bacterial delivery, luminescent activity was recorded by photon collection for one minute under the low light imager. Light emission was noted in the bladder region of the whole animal (Figure 12A). The animals were sacrificed and an abdominal incision was made to expose the bladder. Luminescent activity was positively confirmed to be limited to the bladder (Figure 12B). Upon removal of the bladder from the mice, luminescent
15 activity was no longer visible anywhere in the animals, however, the excised bladders continued to demonstrate light emission (Figure 12C). Based on the results of this experiment, bacteria can target tumors in immunocompetent as well as nude mice. Furthermore, the bacteria can also target smaller tumors.

(F) Bacteria home in on glioma tumors in the brain of rats

20 [0054] Lewis rats with glioma tumors in the brain were intravenously injected with 10^8 attenuated *V. cholera* with the pLITE201 plasmid through the left femoral vein to determine if bacteria can cross the blood-brain barrier and target tumors in immunocompetent animals. The whole animals were monitored for one minute under the low light imager the following day and low levels of luminescent activity was observed through the skull. The rats were sacrificed and
25 the brain tissue was removed in one piece in order to further evaluate the exact location of the luminescent bacteria. Visualization of the excised brain under the imager demonstrated strong luminescent activity in specific regions of the brain (Figure 13A). Similar imaging of control rats without brains tumors, which were intravenously injected with the labeled bacteria, demonstrated absence of any luminescent activity (Figure 13B).

30 (G) Transformed human fibrosarcoma cells home in on subcutaneous glioma tumors in nude mice

[0055] Nude mice with human breast tumors were injected intravenously with 5×10^5 human fibrosarcoma cells, which were permanently transformed with retrovirus derived from pLEIN. Seven days post-injection, the animals were anesthetized Nembutal, and monitored under a fluorescent stereomicroscope. Fluorescent cells were noted only in
35 the tumor region of the whole mice through the skin (Figure 14A1-3). Upon exposure of the tumor tissues by reflection of the overlying skin (Figure 14B1-3), and in cross sections of the tumors (Figure 14C1 -3), fluorescent patches were visible in distinct regions. Close examination of the organs of the mice showed the presence of small clusters of fluorescent cells in the lungs of the animals, demonstrating the affinity of the fibrosarcoma cells for the lungs in addition to the tumorous tissue.

40 **Example 4: Construction of bacterial plasmid vectors that carry the light-emitting protein encoding expression cassettes and the therapeutic gene expression constructs in cis configuration**

(A) Rationale

45 [0056] Using the light-emitting expression systems described above, tumors could be imaged based on light emission for up to 45 days in animals. These findings suggest a remarkable plasmid DNA stability in bacteria in the absence of selection. Therefore, by placing the therapeutic gene cassette in cis configuration with the light-emitting protein expression cassette on the same replicon, light emission can be used as an indicator of therapeutic construct presence
50 and stability.

[0057] In contrast to light-emitting proteins, the therapeutic proteins, endostatin and Pseudomonas exotoxin/TGF alpha fusion protein, are required to be secreted from the bacteria into the medium or into the cytosol of tumor cells for inhibition of tumor growth. To achieve protein secretion from the extracellularly replicating *E. coli* cells into the tumor, two constructs with different signal sequences can be designed. For secretion of endostatin, the *ompF* signal sequence
55 can be placed upstream of the coding sequence of endostatin, which facilitates the secretion into the periplasmic space. To release the endostatin into the medium, an additional protein, the PAS protein, needs to be coexpressed with endostatin. PAS has been shown to cause membrane leakiness and the release of secreted proteins into the medium (Tokugawa et al., J.Biotechnol. 37 (1994), 33; Tokugawa et al., J.Biotechnol. 35 (1994), 69). The second construct for

the secretion of *Pseudomonas* exotoxin/TGF alpha fusion protein from *E. coli* has the OmpA signal sequence upstream of the fusion gene and the release from the periplasmic space into the medium is facilitated by sequences present in domain II of the exotoxin (Chaudhary et al., PNAS 85 (1988), 2939; Kondo et al., J.Biol.Chem. 263 (1988), 9470; Kihara and Pastan, Bioconj.Chem. 5 (1994), 532). To promote secretion of endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein from *L. monocytogenes*, the signal sequence of listeriolysin (LLO) (Mengaud et al., Infect.Immun. 56 (1988), 766) can be placed upstream of each coding sequence.

[0058] For regulation of endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein expression levels in bacteria, vectors can be generated where the therapeutic protein encoding genes are under the control of the T7 promoter or the P_{spac} synthetic promoter (Freitag and Jacobs, Infect.Immun. 67 (1999), 1844). Without exogenous induction, the levels of the therapeutic proteins are low in *E. coli* and in *L. monocytogenes*. The minimal levels of therapeutic proteins in bacteria provide greater safety following intravenous injection of the engineered bacteria. In the following, six newly constructed plasmid DNAs for constitutive and regulated expression of endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein in *E. coli* and *L. monocytogenes* are described. All plasmids to be transferred into *E. coli* will carry the constitutively expressed bacterial *lux* operon, and all the plasmids to be transferred into *L. monocytogenes* will carry the constitutively expressed *sod-gfp* cassette. Plasmids BSPT#1-ESi and BSPT#2-Pti are able to replicate in *E. coli* only, and plasmids BSPT#3, #4, #5, and #6 replicate in *E. coli* and *L. monocytogenes*.

(B) Construction of plasmid vectors for protein expression and secretion from *E. coli*

[0059] The construction of the endostatin secretion vector to be used in *E. coli* is as follows. The coding sequence of human endostatin (591 bp) will be amplified by PCR from the plasmid pES3 with the introduction of the required restriction sites on both ends, followed by ligation into a pBluescript (Clontech Corp., USA) cloning vector to generate pBlue-ES. The ompF signal sequence (Nagahari et al., EMBO J. 4 (1985), 3589) is amplified with Taq polymerase and inserted upstream in frame with the endostatin sequence to generate pBlue-ompF/ES. The expression cassette driven by the T7 promoter will be excised, and inserted into the pLITE201 vector described in Example 1(B), above, carrying the *luxCDABE* cassette, to produce the plasmid pLITE-ompF/ES. The sequence encoding the PAS factor (a 76 amino acid polypeptide) will be amplified from the chromosomal DNA of *Vibrio alginolyticus* (formerly named *Achromobacter iophagus*) (NCIB 11038) with Taq polymerase using the primers 5'-GGGAAAGACATGAAACGCTTA3-' and 5'-AAACAACGAGTGAATTAGCGCT-3', and inserted into the multiple cloning sites of pCR-Blunt (Clontech Corp., USA) to create the expression cassette under the control of the lac promoter. The resulting plasmid will be named pCR-PAS. The lac promoter linked to the pas gene will be excised from pCR-PAS and inserted into pLITEompF/ES to yield the final plasmid BSPT#1-ESi.

[0060] Plasmid pVC85 (Pastan, see above) contains a T7 promoter, followed by an ompA signal sequence, and a sequence encoding domain II and III of *Pseudomonas* exotoxin (PE40). The DNA sequence encoding PE40 will be excised with restriction enzymes and replaced with a fragment of PE37/TGF alpha (*Pseudomonas* exotoxin A 280-613/TGF alpha) obtained from the plasmid CT4 (Pastan, see above) to create the plasmid pVC85-PE37/TGF alpha. The expression cassette of ompAPE37/TGF alpha linked to the T7 promoter will be excised and inserted into pLITE201 to yield the final plasmid BSPT#2-PTI.

(C) Construction of plasmid vectors for protein expression and secretion from *L. monocytogenes*

[0061] Genes encoding endostatin or PE37/TGF alpha will be inserted downstream of the listeriolysin (LLO) signal sequence in the plasmid pCHHI to generate pCHHI-ES and pCHHI-PE37/TGF alpha. Constitutive expression of the therapeutic proteins will be obtained by linking the above secretion cassettes to the listeriolysin promoter obtained from the pCHHI vector. The SOD-GFP expression cassette, excised from the plasmid pSOD-GFP (Götz et al. PNAS in press.) will be inserted into pCHHI-ES to generate BSPT#3-ESc, and into pCHHI-PE37/TGF alpha to generate BSPT#4-PTc. For the expression of the therapeutic proteins under the control of an IPTG inducible promoter, the listeriolysin promoter in BSPT#3-ESc and BSPT#4-PTc will be replaced with the P_{spac} promoter from the plasmid pSPAC (Yansura and Henner, PNAS USA 81 (1984), 439) to generate BSPT#5-ESi and BSPT#6-PTi. P_{spac} is a hybrid promoter consisting of the *Bacillus subtilis* bacteriophage SPO-1 promoter and the lac operator. IPTG-induced GFP expression from the P_{spac} promoter has been documented in *L. monocytogenes* in the cytosol of mammalian cells.

Example 5: Demonstration of the expression of luciferase and GFP in bacteria and verification of the secretion of endostatin and recombinant toxin/TGF alpha fusion protein and their function in cell culture assays

[0062] To be able to detect the presence of *E. coli* and *L. monocytogenes* in tumor tissues in live animals, the levels of the constitutively expressed luciferase and GFP in bacteria need to be adequate. Therefore, after transformation of recipient *E. coli* or *L. monocytogenes* with the constructs described in Example 4, the colonies with the highest luciferase

light emission or OFP fluorescence will be selected. In addition to characterizing light emission from each selected colony before intravenous injection, the ability of the selected transformants to secrete endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein into the medium needs to be confirmed. The presence of endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein synthesized within *E. coli* and *L. monocytogenes* will be determined by extracting these proteins from the cell pellet. The secreted proteins in the medium will be concentrated and analyzed by gel separation and the quantity will be determined by Western blotting. It is imperative to determine the percentage of the newly synthesized proteins expressed from each plasmid construct in either *E. coli* or *L. monocytogenes* that is present in the medium. It is also essential to confirm, in addition to constitutive expression of endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein, that expression can be induced in *E. coli* and in *L. monocytogenes* upon the addition of IPTG to the bacterial culture medium. For the design of future tumor therapy protocols, the relative amounts of protein secreted by the constitutive expression system needs to be compared to the induced expression levels for a defined time period first in bacterial cultures. It is equally essential to determine that both proteins when synthesized in *E. coli* and *L. monocytogenes* are biologically active if generated from the proposed constructs. Both proteins were synthesized previously in *E. coli* and were shown to be active.

[0063] The results of the experiments described below should confirm whether endostatin is successfully secreted from *E. coli* using the OmpF signal peptide in combination with PAS pore forming protein expression. These experiments will also show if the PE40/TGF alpha and PE37/TGF alpha fusion proteins are secreted from bacteria using the OmpA signal peptide in combination with domain II of PE. Further, the listeriolysin signal peptide may also facilitate the secretion of endostatin and the chimeric toxin/TGF alpha fusion protein into the medium as well as into the cytosol of infected tumor cells. Using the migration inhibition assay and the protein synthesis inhibition assay, it can be expected to determine that both proteins secreted into the medium are biologically active. The presence and quantities of these proteins may be regulated by replacing the constitutive promoters with promoters that can be induced by IPTG.

[0064] In addition to the secretion system described below, alternative secretion systems such as the *E. coli* HlyBD-dependent secretion pathway (Schlor et al., Mol.Gen.Genet. 256 (1997), 306), may be useful. Alternative secretion signals from other gram positive bacteria, such as the *Bacillus* sp. endoxylanase signal peptide (Choi et al., Appl. Microbiol.Biotechnol. 53 (2000), 640; Jeong and Lee, Biotechnol.Bioeng. 67 (2000), 398) can be introduced.

(A) Confirmation of endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein secretion from bacteria into growth medium

[0065] *E. coli* strains (DH5α and BL21(λDE3)) will be transformed with BSPT#1-ESi and BSPT#2-PTi plasmid DNA. *L. monocytogenes* strain EGDA2 will be transformed with plasmids BSPT#3-ESc, BSPT#4-PTc, BSPT#5-ESi, and BSPT#6-PTi individually. After plating on appropriate antibiotic-containing plates, individual colonies will be selected from each transformation mixture. These colonies will be screened under a low light imager and fluorescence microscope for luciferase and GFP expression, respectively. Three colonies with the most intense light emission from each transformation batch will be chosen for further studies. To verify the secretion of endostatin and *Pseudomonas* exotoxin/TGF alpha fusion protein from each selected transformant, the cells will be grown in minimal medium to log phase. After centrifuging down the bacteria, the supernatants will be passed through a 0.45-μm-pore-size filter, and the bacterium-free medium will be used for precipitation of the secreted proteins. The precipitates will be collected by centrifugation. Pellets will be washed, dried, and re-suspended in sample buffer for protein gel separation. Proteins from aliquots corresponding to 10 μl of bacterial culture will be compared to proteins from 200 μl of culture supernatant after separation in a 10% SDS-polyacrylamide gel. Western blot analysis will be performed using polyclonal antibody against endostatin (following the antibody production protocol described by Timpl, Methods Enzymol. 82 (1982), 472) and monoclonal antibody against TGF alpha (Oncogene Research Products, Cambridge, MA, USA). The optimal growth conditions will be established for secretion by sampling the growth medium at different times during growth. A similar method has been used previously to analyze secreted proteins in *Salmonella typhimurium* culture supernatant (Kaniga et al., J.Bacteriol. 177 (1995), 3965). By use of these methods the amount of secreted proteins in the bacterial culture medium generated by each of the constructs without induction will be established. To estimate the increase in the amount of secreted proteins in the medium, IPTG-dependent promoter activation experiments will be carried out by adding IPTG to the bacterial culture in log phase for 3 to 6 hours, and the secreted proteins will be assayed as above.

(B) Verification of the biological activity of endostatin secreted by *E. coli* and *L. monocytogenes* using a migration inhibition assay

[0066] It has been shown that endostatin inhibits vascular endothelial growth factor (VEGF)-induced human umbilical vein endothelial cell (HUVEC) migration. Thus, the biological activity of endostatin secreted by bacteria can be tested using the HUVEC migration assay provided by Cascade Biologics, Portland, OR. The inhibition of cell migration will be assessed in 48-well chemotaxis chambers (Neuro Probe, Gaithersburgs, MD) (Polverine et al., Methods Enzymol.

EP 1 281 767 A2

198 (1991), 440). Bacterium-free supernatant from each secretion construct will be added to HUVECs for preincubation for 30 min. After incubation, the HUVECs will be placed in the upper chamber. The migration of HUVECs into the lower chamber induced by VEGF₁₆₅ (R&D Systems, Minneapolis, NIN) will be quantified by microscopic analysis. The concentration of functional endostatin in the medium will be directly proportional to the degree of inhibition of HUVEC migration.

(C) Testing the cytotoxic activity of secreted recombinant PE toxin tumor in tumor cell cultures

[0067] The inhibitory activity of the chimeric toxin in mammalian cells will be measured based on inhibition of de novo protein synthesis by inactivating EF-2 (Carroll and Collier, J.Biol.Chem. 262 (1987), 8707). Aliquots of bacterium-free supernatants obtained from the expression of various recombinant PE secretion constructs in *E. coli* and in *L. monocytogenes* will be added to the C6 glioma cells or to HCT116 colon carcinoma cells. Following treatment with medium, the mammalian cells will be pulsed with [³H]-leucine, and the incorporation will be determined in the protein fraction. To determine the presence of secreted chimeric toxin proteins in *L. monocytogenes*-infected mammalian cells, the bacteria will be eliminated from the medium by gentamicin treatment. The mammalian cells containing *L. monocytogenes* in the cytosol will be lysed, and the released bacteria removed from the lysate by filtration. The mammalian cell lysate containing the secreted chimeric toxins will be assayed in protein synthesis inhibition experiments. The inhibition of [³H]-leucine incorporation in tumor cell culture will be directly proportional to the amount of the biologically active chimeric toxin protein in the medium and cell lysate.

Example 6: Determination of the entry, localization and distribution of intravenously injected bacteria in tumors of live animals

(A) Rationale

[0068] Since only a small number of intravenously injected bacteria escape the immune system by entering the tumor, their immediate localization is not possible due to limited light emission in live animals. Their location can only be verified by sectioning the tumor to identify the early centers of light emission. Looking at sections at a later time point, bacteria can be seen throughout the entire tumor due to rapid replication. To determine whether one or multiple bacteria enter through the same site, red fluorescent protein can be used to label the extracellularly replicating *E. coli* and green fluorescent protein for the intracellularly replicating *L. monocytogenes*. By visualizing the distribution of the red and green fluorescence in tissue sections, the entry sites as well as replication and localization of *E. coli* and *L. monocytogenes* can be determined individually and simultaneously in the central or peripheral regions of the tumor. It can be expected that the patterns of entry and distribution obtained in implanted tumors mimic those of spontaneous tumors, accordingly, the bacterium-based diagnosis and protein therapy will become a valid approach.

[0069] With the experiments described in section (B), below, the entry, replication, and distribution of light-emitting bacteria in spontaneous tumors can be compared to the distribution patterns in implanted tumors. Further, double-labeling experiments will allow the operator to precisely locate the extracellularly replicating *E. coli* and the intracellularly replicating *L. monocytogenes* in the same tumor sections. Lastly, it can be determined (subsequent to a five-day bacterial colonization) whether bacteria are distributed evenly in the tumors or preferential localization occurs in the periphery of the tumor or in the necrotic center. A possible reduction in bacterial entry into spontaneously occurring tumors due to the immunocompetence of these animals can be overcome by increasing the number of intravenously injected bacteria.

(B) Intravenous injection of *E. coli* expressing red fluorescent protein and *L. monocytogenes* expressing green fluorescent protein into nude mice and into rodents with implanted and spontaneous tumors

[0070] *E. coli* (DH5 α) carrying the DsRed (Matz et al., Nat.Biotech. 17 (1999), 969) expression cassette under the control of a constitutive promoter are used in this experiment. *L. monocytogenes* EGD strain derivatives with in-frame deletion in each of the virulence genes were individually labeled with the green fluorescent protein cassette driven by the constitutive SOD promoter.

[0071] The localization and intratumoral distribution of bacteria will first be studied in nude mice with implanted C6 glioma or HCT116 colon carcinoma tumors. C6 glioma or HCT116 colon carcinoma cells (5×10^5 in 100 μ l) will be subcutaneously injected into the right hind leg of the animals. Twelve days after tumor cell injection, the animals will be anesthetized, and the left femoral vein surgically exposed. Light-emitting bacteria (1×10^6 cells re-suspended in 50 μ l of saline) will be intravenously injected, and the wound incision will be closed with sutures. Tumors will be measured three times a week using a caliper. Tumor volume will be calculated as follows: small diameter x large diameter x height / 2.

EP 1 281 767 A2

[0072] Intracerebral glioma tumors will be generated by injecting C6 glioma cells into the head of Wistar rats. Rats will be anesthetized with Ketamine (70-100 mg/kg body weight) and Xylazine (8-10 mg/kg body weight). A midline scalp incision (0.5 - 1 cm) will be made, skin will be reflected, and a 1 mm burr hole will be made in the skull located 2 mm to the left and 2.5 mm posterior to the bregma. Tumor cells will be pipetted into an insulin syringe fitted with a 29-gauge needle and mounted in a stereotactic holder. The needle will be inserted vertically through the burr hole to a depth of 3 mm. After injection into the brain of a 5 µl volume of either 5 x 10⁵ C6 cells or PBS as control, the needle will be kept in place for 15 sec and then withdrawn. The skin incision will be closed with surgical clips. Ten days after cell injection, an intracranial glioma will develop which is 5-10 mm in diameter. The same protocols involving intravenous injection of bacteria into animals with tumors will be followed through the remainder of the proposal.

[0073] The localization of bacteria in the tumor, based on GFP or RFP, will also be analyzed using cryosectioned tumor tissues. A reliable morphological and histological preservation, and reproducible GFP or RFP detection may be obtained using frozen sections after a slow tissue freezing protocol (Shariatmadari et al., *Biotechniques* 30 (2001), 1282). Briefly, tumor tissues will be removed from the sacrificed animals to a Petri dish containing PBS and dissected into the desired size. The samples will be mixed for 2 h in 4% paraformaldehyde (PFA) in PBS at room temperature. They will be washed once with PBS, and embedded in Tissue-Tek at room temperature, and then kept in the dark at 4°C for 24 h and slowly frozen at -70°C. Before sectioning, the tissue will be kept at -20°C for 30 min. Then, 10- to 50-µm-thick sections will be cut with a Reichert-Jung Cryocut 1800 cryostat and collected on poly-L-lysine (1%)-treated microscope slides. During sectioning, the material will be kept at room temperature to avoid several freezing and thawing cycles. Finally, the sections will be rinsed in PBS and mounted in PBS and kept in the dark at 4°C.

[0074] To monitor the entry of light emitting *E. coli* and *L. monocytogenes* from the blood stream into the tumor, 27 nude mice will be injected with C6 tumor cells, and 27 nude mice with HCT116 colon carcinoma cells. Twelve days after tumor development, 9 animals from the C6 group and 9 from the HCT116 group will receive an intravenous injection of *E. coli* with the RFP construct. Another 9 animals from each group will receive an intravenous injection of *L. monocytogenes* transformed with the GFP construct. The third group of 9 animals from each tumor model will receive both *E. coli* and *L. monocytogenes* (1 x 10⁶ cells of each). Five hours, 25 hours, and 5 days after injection, three animals of each treatment group will be sacrificed, their tumors excised, and processed individually as described in the above cryosectioning protocol. After freezing, each tumor will be cut into two halves. One half of the tumor will be used for preparing thick sections (60-75 µm), which will be analyzed under a fluorescence stereomicroscope to observe the distribution of bacteria in the sections of tumors obtained from each time point of the experiment. The regions of interest will be identified, thin sectioned, prepared, and analyzed with laser scanning cytometry and under the confocal microscope followed by image reconstruction.

[0075] In parallel experiments, animals with spontaneous tumors, as listed in Table 1, will be obtained and used in intravenous injection experiments with *E. coli* carrying the bacterial *lux* operon. Two animals of each tumor model will be used, and the luciferase light emission monitored daily under the low light imager. It is expected that the spontaneously occurring tumors can be imaged similarly to the implanted tumors based on bacterial luciferase expression. Two of the spontaneous tumor models, mice with adenocarcinoma of the large intestine and mice with adenocarcinoma of the mammary tissue, will be used for bacterial localization experiments following intravenous injection of *E. coli* expressing RFP and *L. monocytogenes* expressing GFP as described above. It can be expected that these experiments will emphasize the significance of the bacterium-based diagnosis and protein therapy system.

Table 1

Spontaneous tumor animal models				
Animal species	Strain name	Tumor description	Source	Reference
Mouse	129/Sv-Madh3 ^{tm1par}	spontaneous adenocarcinoma of large intestine	Jackson Laboratories Bar Harbor, ME	Zhu et al., <i>Cell</i> <u>94</u> (1988), 703
Mouse	FVB/N-TgN (UPII-SV40T)29Xrw	spontaneous carcinoma of bladder with metastasis to the liver	Jackson Laboratories Bar Harbor, ME	Zhang et al., <i>Cancer Res.</i> <u>59</u> (1999), 3512
Mouse	FVB-neuN(N#202)	spontaneous adenocarcinoma of mammary tissue	Jackson Laboratories Bar Harbor, ME	Guy et al., <i>PNAS USA</i> <u>89</u> (1992), 10578
Rat	F344/CrCr1BR	spontaneous carcinoma of pituitary	Charles River Laboratories, Wilmington, MA	Hosokawa et al., <i>Toxicol. Pathol.</i> <u>21</u> (1993), 283

Example 7: Verification of bacterium-mediated tumor targeting and bacterium-secreted protein therapy in rodents with implanted or spontaneous tumors

(A) Rationale

[0076] As shown in the previous examples, intravenous injection of light-emitting bacteria results in entry, replication, and accumulation only in the tumor regions in animals. This process can be monitored by imaging of light emission in tumors. Placing the endostatin and chimeric toxin expressing gene cassettes in cis configuration with a light-emitting gene cassette provides an indirect detection system in vivo for their temporal and spacial delivery via bacteria.

[0077] The endostatin and chimeric toxin gene cassettes are linked to signal peptide encoding sequences, which facilitate the secretion of these proteins into the extracellular space in the tumor or into the cytosol of infected tumor cells. Both proteins secreted from bacteria into the extracellular space of the tumor are expected to function similarly to directly injected purified proteins. Both proteins secreted from *L. monocytogenes* into the cytosol of the infected tumor cells will resemble the viral delivery system reported earlier for endostatin. The bacterial systems can be used as a constitutive secretion system or as an exogenously added IPTG-activatable secretion system in the tumor. By regulating the expression levels of the therapeutic proteins in bacteria that colonize the tumor, the secreted amount of proteins inhibiting tumor growth can be determined. Without the addition of IPTG, the inhibitory protein secretion from the intravenously injected bacteria will be kept at minimum while in blood circulation. This will provide an added safety to the recipient tumorous animals during delivery of bacteria. Using the BSPT system, the onset and duration of the therapy can be controlled by the addition of IPTG. Upon completion of the treatment, the bacterial delivery system can be eliminated by administration of antibiotics, similar to treating a bacterial infection.

(B) Determination of the effect of endostatin and Pseudomonas exotoxin/TGF alpha fusion protein secreted by *E. coli* and *L. monocytogenes* on tumor growth in animals with implanted tumors

[0078] The inhibitory effect of endostatin and the cytotoxicity of the chimeric toxin secreted by *E. coli* and *L. monocytogenes* in tumors will be determined as follows. Thirty-five nude mice bearing 10-day-old C6 tumors will be injected with bacterial constructs as follows: (a) Five mice with *E. coli* engineered to secrete endostatin; (b) Five mice with *E. coli* engineered to secrete chimeric toxin; (c) Five mice with *L. monocytogenes* engineered to secrete endostatin; (d) Five mice with *L. monocytogenes* engineered to secrete chimeric toxin; (e) Five mice with *E. coli* secreting endostatin and chimeric toxin; (f) control group: five mice injected with *E. coli* expressing bacterial luciferase alone, and five mice with *L. monocytogenes* expressing GFP. At the time of bacteria injection, each tumor volume will be determined. Three days after injection, the replication of bacteria in the tumors will be monitored under a low light imager or under a fluorescence stereomicroscope. The light emission and the tumor volume will be measured daily up to 20 days after bacterial injection. Ten days after injection, one animal from each group will be sacrificed and the levels of the secreted proteins present in the tumor tissue will be analyzed using Western blot analysis. These experiments will result in inhibition of tumor growth in endostatin treated animals or a more dramatic tumor regression in animals treated with chimeric toxin proteins. The tumor growth in control animals is not expected to be affected by the bacteria alone.

[0079] In a follow-up experiment, mice with spontaneous adenocarcinoma of mammary tissue (strain FVB-neuN (N#202), Table 1) will be used to study the effect of secreted proteins on tumor growth. An experimental scheme identical to that described for the C6 tumor analysis will be used. At the completion of tumor therapy, the presence of endostatin or chimeric toxin in the tumor tissue will be determined by Western blot analysis. An identical experimental design will be used to assay the effect of IPTG-induction of endostatin and chimeric toxin production in bacteria in C6 tumors as well as in the spontaneously occurring breast tumor mouse model. It is expected that multiple IPTG induction of protein expression in bacteria might be required for successful tumor therapy.

[0080] At any stage of tumor treatment, it may be required to remove the light emitting and therapeutic gene containing bacteria from the animal. To carry out this experiment, mice with 12-day-old C6 tumors will be intravenously injected with *E. coli* expressing the bacterial luciferase. Three days after injection, antibiotic therapy will be initiated by intraperitoneal administration of gentamicin (5 mg/kg body weight) twice daily, or the newly discovered clinafloxacin (CL960) (Nichterlein et al., Zentralbl.Bakteriol. 286 (1997), 401). This treatment will be performed for 5 days, and the effect of antibiotics on the bacteria will be monitored by imaging light emission from the animals daily.

[0081] By completing the above experiments, it is expected that endostatin and chimeric toxin proteins secreted into the tumors will cause the inhibition of tumor growth and measurable tumor regression. It is anticipated that tumor regression will be achieved in both groups of rodents with implanted tumors and with spontaneously occurring tumors. Experiments with simultaneous application of secreted endostatin and chimeric toxin proteins in tumor treatment may give the most promising results. The removal of the engineered bacteria from the tumor by administration of antibiotics is an added safety measure of the bacterium-secreted protein therapy (BSPT) of the present invention.

EP 1 281 767 A2

Claims

1. A diagnostic or pharmaceutical composition comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal.
2. The diagnostic or pharmaceutical composition of claim 1, wherein the protein capable of inducing a detectable signal is a luminescent and/or fluorescent protein.
3. The diagnostic or pharmaceutical composition of claim 1, wherein the protein capable of inducing a detectable signal is a protein inducing a signal detectable by magnetic resonance imaging (MRI) or capable of binding a contrasting agent, chromophore or a ligand required for visualization of tissues..
4. The diagnostic or pharmaceutical composition of any one of claims 1 to 3, wherein the microorganism is a bacterium or a virus.
5. The diagnostic or pharmaceutical composition of claim 4, wherein the virus is *Vaccinia* virus.
6. The diagnostic or pharmaceutical composition of claim 4, wherein the bacterium is attenuated *Salmonella typhimurium*, attenuated *Vibrio cholerae*, attenuated *Listeria monocytogenes* or *E.coli*.
7. The diagnostic or pharmaceutical composition of claim 1, wherein the cell is a mammalian cell.
8. The diagnostic or pharmaceutical composition of any one of claims 1 to 7, wherein the luminescent or fluorescent protein is luciferase, RFP or GFP.
9. The diagnostic or pharmaceutical composition of any one of claims 1 to 8, wherein the microorganism or cell additionally contains a gene encoding a substrate for a luciferase.
10. Use of the microorganism or cell as defined in any one of claims 1 to 9 for the preparation of a diagnostic composition for tumor-imaging or monitoring a therapeutic tumor treatment.
11. Use according to claim 10, wherein tumor-imaging or monitoring is carried out by MRI.
12. A pharmaceutical composition containing a microorganism or cell as defined in any one of claims 1 to 9, wherein said microorganism or cell furthermore contains one or more expressible DNA sequences encoding (a) protein(s) suitable for tumor therapy and/or elimination of metastatic tumors.
13. The pharmaceutical composition of claim 12, wherein the protein suitable for tumor therapy and/or elimination of metastatic tumors is endostatin or recombinant chimeric toxin PE37/transforming growth factor alpha (TGF-alpha).
14. The pharmaceutical composition according to claim 12 or 13, wherein the expressible DNA sequences are on a BAC, MAC, cyber cell or cyber virus.
15. Use of the microorganism or cell as defined in any one of claims 12 to 14 for the preparation of a pharmaceutical composition for tumor therapy and/or elimination of a metastatic tumor.
16. Use according to claim 15, wherein the tumor is a bladder tumor, breast tumor, prostate tumor, glioma tumor, liver tumor, skin tumor, adenocarcinoma, ovarian carcinoma or pancreatic carcinoma.

Fig. 1.

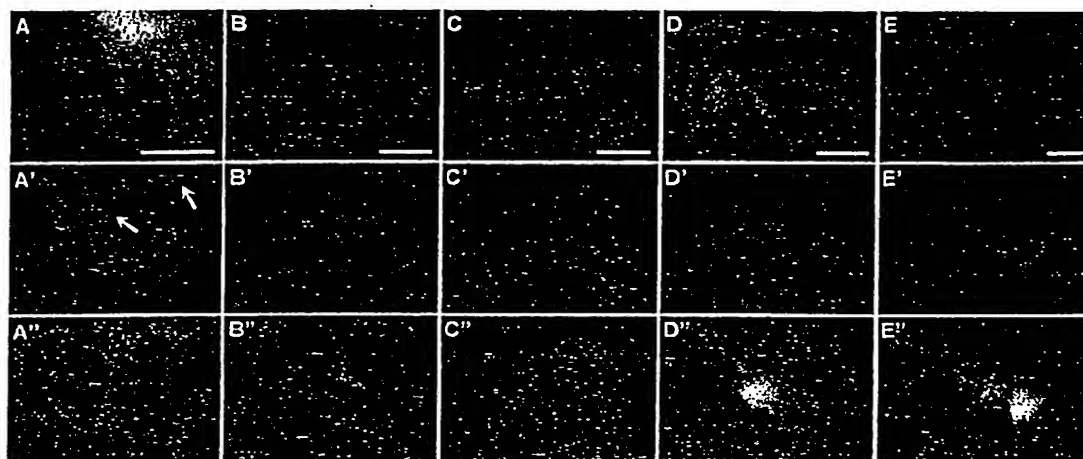


Fig. 2.



Fig. 3.

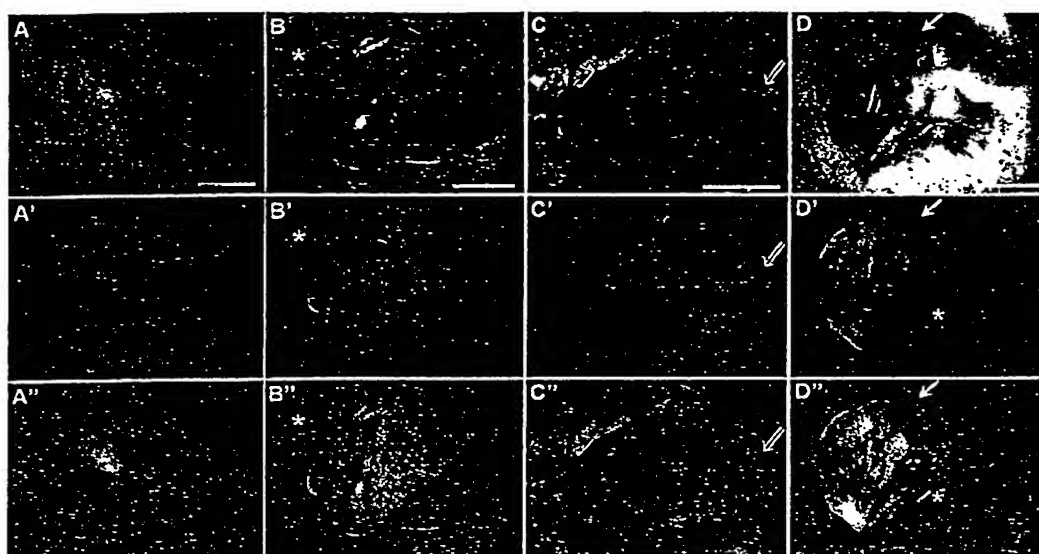


Fig. 4.

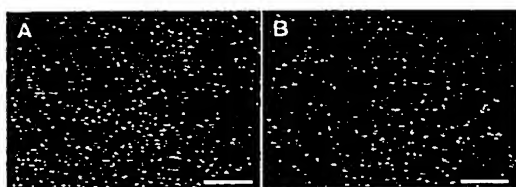


Fig. 5.

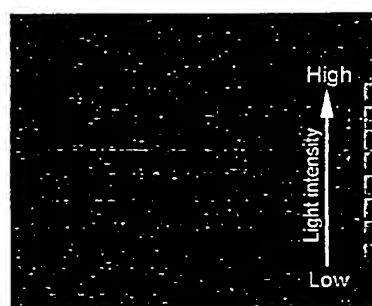


Fig. 6.

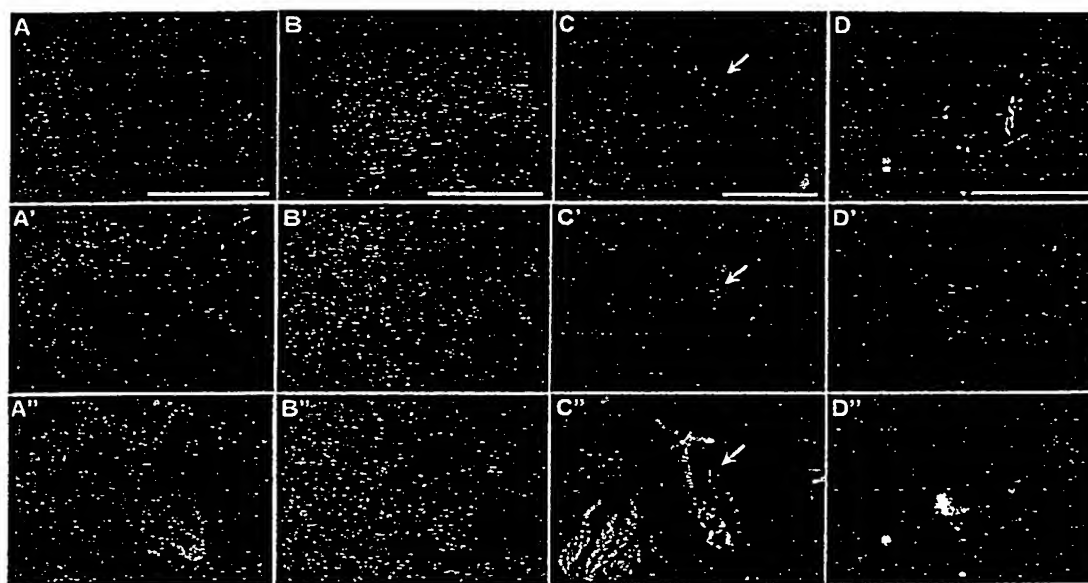
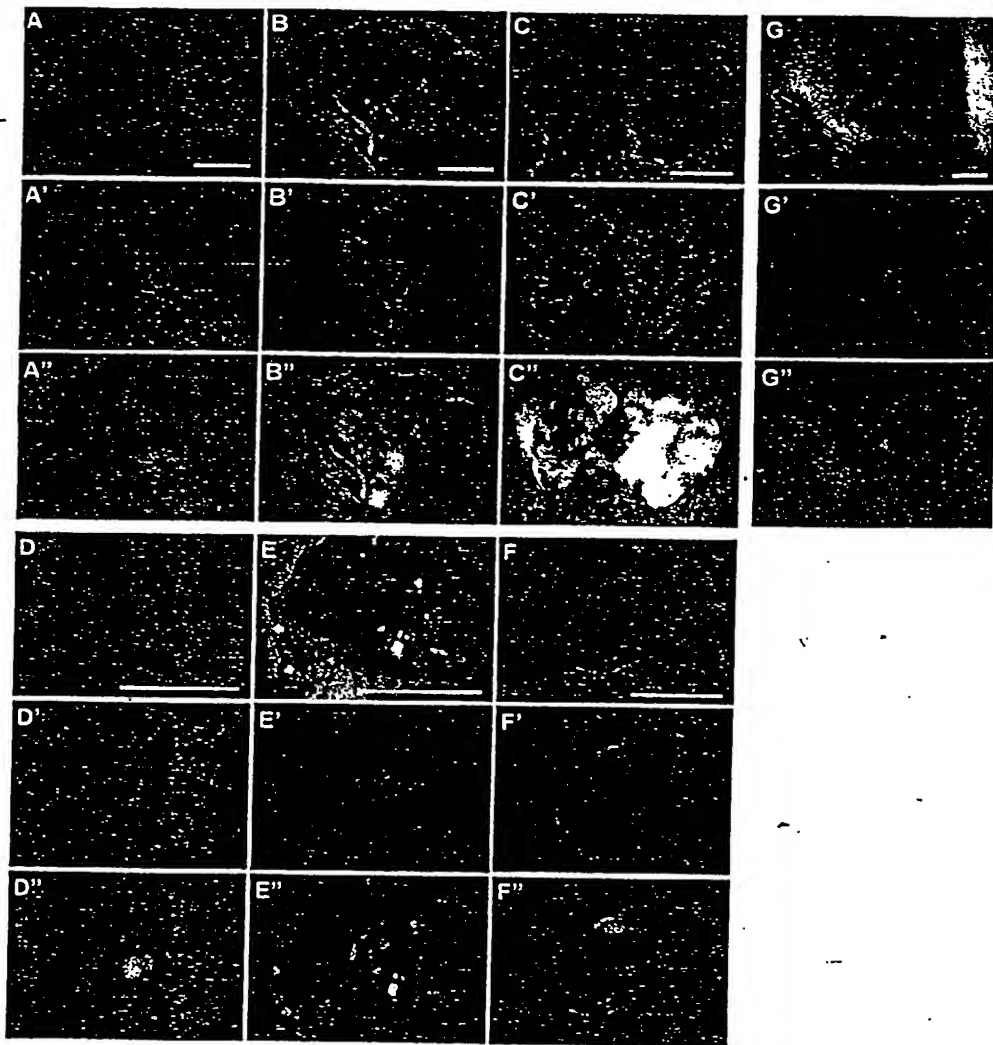


Fig. 7.



EP 1 281 767 A2

Fig. 8

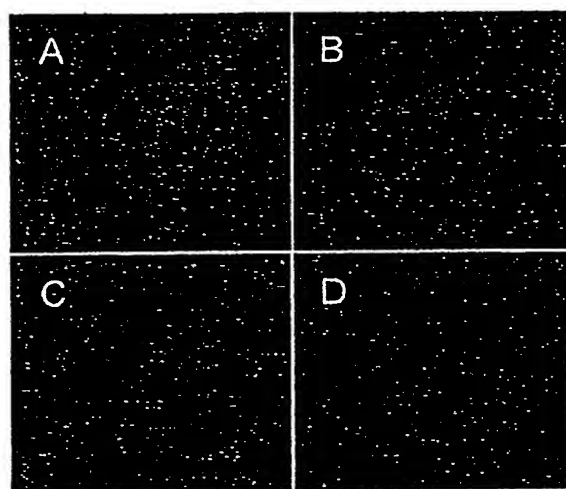


Fig. 9

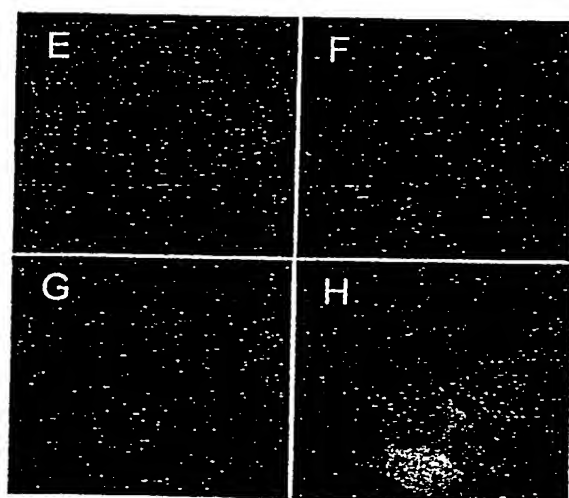
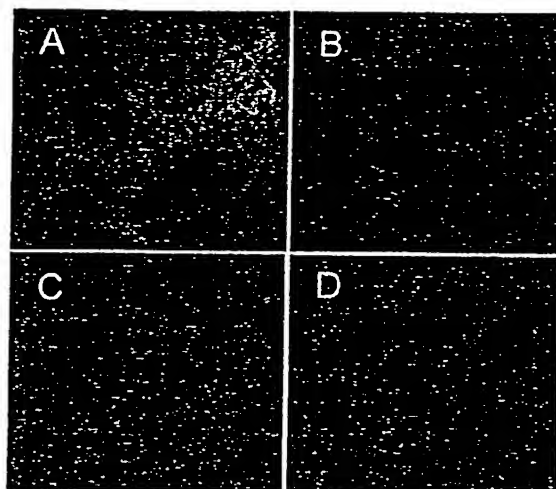


Fig. 10

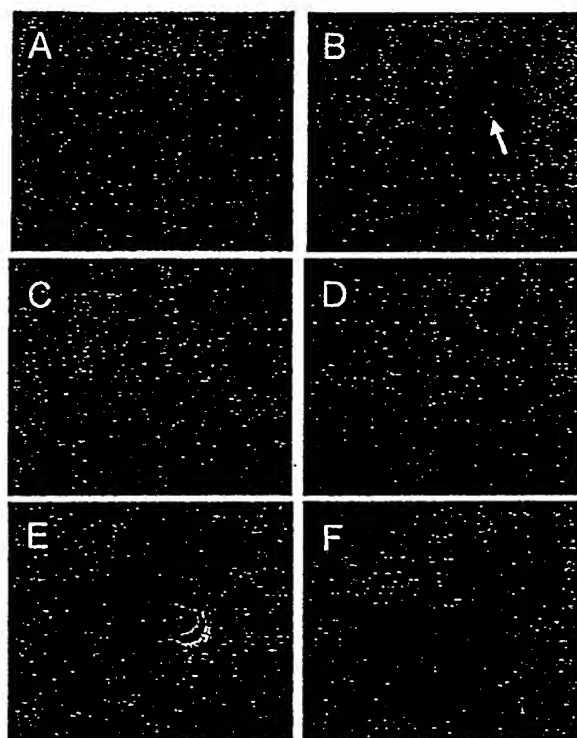


Fig. 11

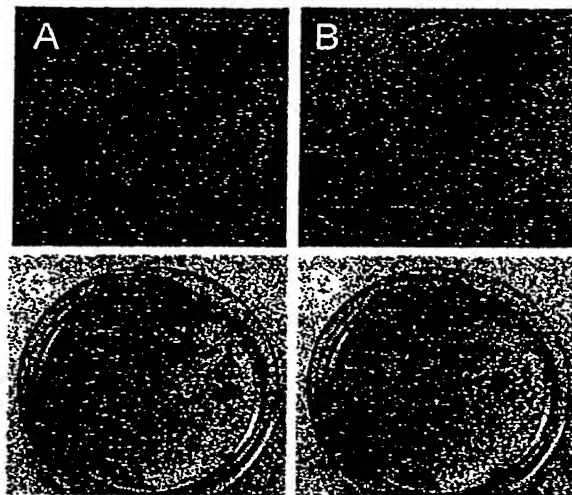


Fig. 12

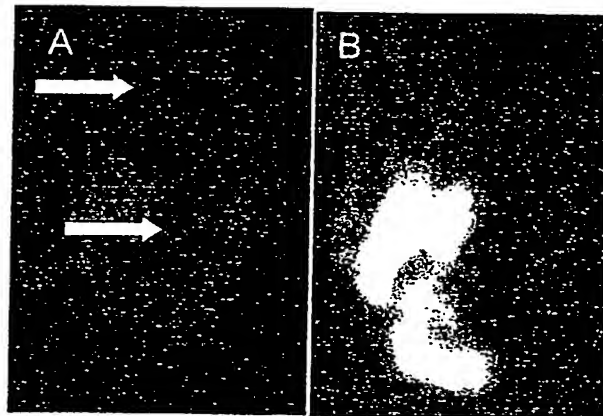


Fig. 13

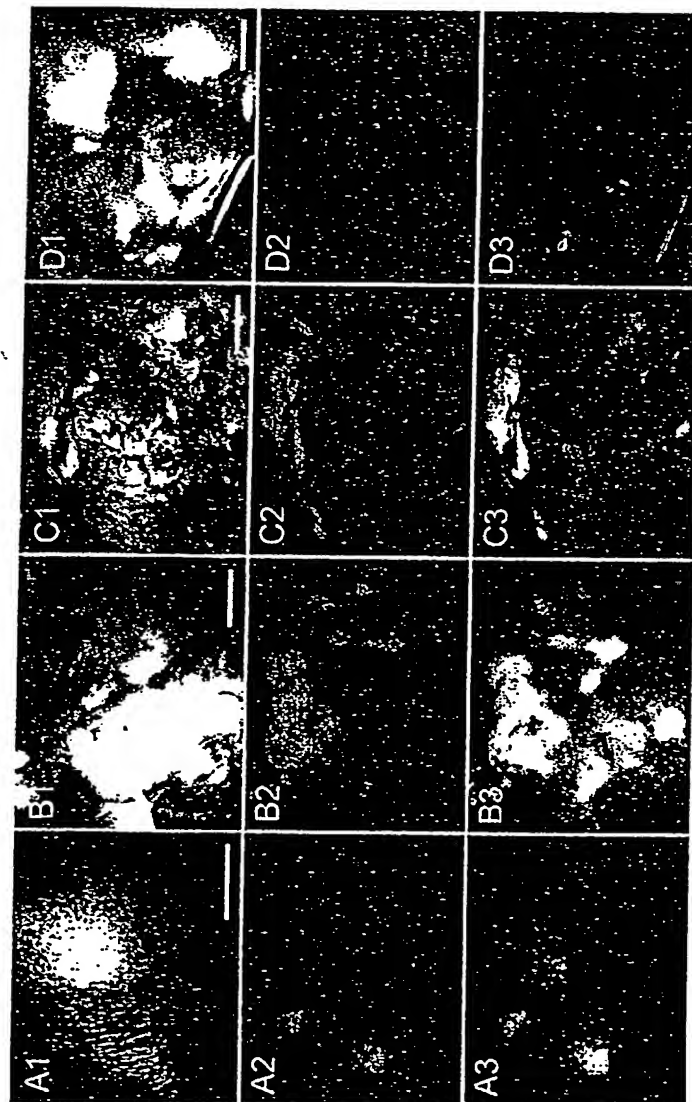
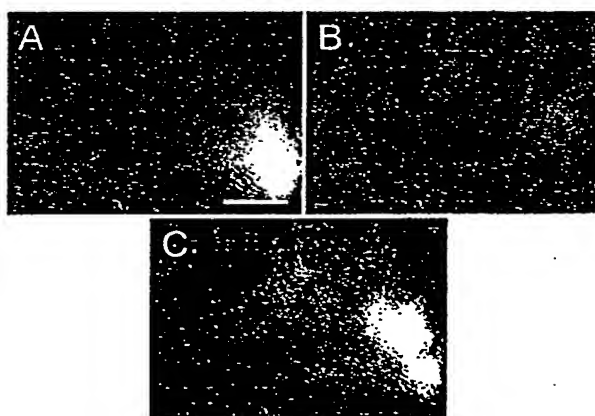
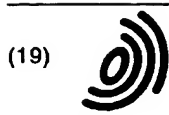


Fig. 14





Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 281 767 A3**

(12) **EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:
28.05.2003 Bulletin 2003/22

(51) Int Cl.7: **C12Q 1/66, C12Q 1/02,**
A61K 49/00, C12N 15/03

(43) Date of publication A2:
05.02.2003 Bulletin 2003/06

(21) Application number: **01125911.6**

(22) Date of filing: **30.10.2001**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(72) Inventor: **Szalay, Aladar A.**
Highland, CA 92346-6213 (US)

(74) Representative: **Schüssler, Andrea, Dr.**
Kanzlei Huber & Schüssler
Truderinger Strasse 246
81825 München (DE)

(30) Priority: **31.07.2001 EP 01118417**

(71) Applicant: **Szalay, Aladar A.**
Highland, CA 92346-6213 (US)

(54) **Light emitting microorganisms and cells for diagnosis and therapy of tumors**

(57) Described are diagnostic and pharmaceutical compositions comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal, e. g. a luminescent or fluorescent protein. Moreover, the use of said microorganism or cell for tumor-targeting or

tumor-imaging is described. For therapeutic uses, said microorganism or cell additionally contain an expressible DNA sequence encoding a protein suitable for tumor therapy, e.g. a cytotoxic or cytostatic protein.

EP 1 281 767 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 12 5911

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 01 25399 A (SLOAN KETTERING INST CANCER ;VION PHARMACEUTICALS INC (US)) 12 April 2001 (2001-04-12) * the whole document * * page 4, line 8 - line 24 * * page 9, line 35 - line 36 * * page 18, line 21 - page 19, line 7 * * page 42, line 24 - page 53, line 33 * * page 54, line 32 - page 56, line 1 * * page 62, line 32 - page 64, line 8 * * claims 1-5,7,16,34-37,55-58,60-67 *	1,2,4,6, 8,10,12, 15,16	C12Q1/66 C12Q1/02 A61K49/00 C12N15/03
Y	---	1,12,13	
X	WO 01 18195 A (XENOGEN CORP) 15 March 2001 (2001-03-15) * abstract * * examples 6,7 * * page 26, line 24 - page 27, line 14 * * page 28, line 29 - page 29, line 33 *	1,2,4, 6-9	
X	RODRIGUEZ J F ET AL: "EXPRESSION OF THE FIREFLY LUCIFERASE GENE IN VACCINIA VIRUS A HIGHLY SENSITIVE GENE MARKER TO FOLLOW VIRUS DISSEMINATION IN TISSUES OF INFECTED ANIMALS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 85, no. 5, 1988, pages 1667-1671, XP002194182 1988 ISSN: 0027-8424 * abstract * * the whole document * ---	1,2,4,5, 8,9	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12Q A61K C12N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 4 April 2003	Examiner Tuytman, A
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03.02 (P04C01)



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 12 5911

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 01 05229 A (BRIGHAM & WOMENS HOSPITAL ;CHIOCCA E ANTONIO (US); GEN HOSPITAL CO) 25 January 2001 (2001-01-25) * claims 1-16 * * page 16, line 7 - page 17, line 17 * * page 21, line 27 - page 22, line 6 * * page 22, line 19 - line 25 * * the whole document *	1,2,4,7,8,10,12,14,15	
X	LOUIE ANGELIQUE Y ET AL: "In vivo visualization of gene expression using magnetic resonance imaging" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 18, March 2000 (2000-03), pages 321-325, XP002181437 ISSN: 1087-0156 * the whole document * * page 321, right-hand column, line 10 - line 24 *	1,3,11	
X	WEISSLEDER RALPH ET AL: "In vivo magnetic resonance imaging of transgene expression." NATURE MEDICINE, vol. 6, no. 3, March 2000 (2000-03), pages 351-354, XP002194183 ISSN: 1078-8956 * the whole document * * page 351, left-hand column, line 31 - right-hand column, line 19 * * figure 1 *	1,3,11	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 4 April 2003	Examiner Tuynman, A
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

EPO FORM 1503 03/02 (P0401)



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 12 5911

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	TJUVAJEV J ET AL: "Salmonella-based tumor-targeted cancer therapy: tumor amplified protein expression therapy (TAPET(TM)) for diagnostic imaging" JOURNAL OF CONTROLLED RELEASE, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, NL, vol. 74, no. 1-3, 6 July 2001 (2001-07-06), pages 313-315, XP004297541 ISSN: 0168-3659 * the whole document *	1,12,15,16	
Y	CLAIRMONT C ET AL: "Enhanced antitumor activity from tumor-targeting Salmonella expressing endostatin." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL, no. 41, March 2000 (2000-03), page 732 XP008015903 91st Annual Meeting of the American Association for Cancer Research.; San Francisco, California, USA; April 01-05, 2000, March, 2000 ISSN: 0197-016X * the whole document *	1,12,13	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
Place of search THE HAGUE		Date of completion of the search 4 April 2003	Examiner Tuynman, A
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03.02 (P4/C01)

EP 1 281 767 A3

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 01 12 5911

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

04-04-2003

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0125399	A	12-04-2001	AU	7993600 A	10-05-2001
			WO	0125399 A2	12-04-2001
WO 0118195	A	15-03-2001	AU	7126600 A	10-04-2001
			EP	1212429 A2	12-06-2002
			WO	0118195 A2	15-03-2001
WO 0105229	A	25-01-2001	WO	0105229 A1	25-01-2001

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.